Cross-linking of Knitted Silk Scaffold for Effective Entheseal Chondrogenesis and Ligamentogenesis

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Cross-linking of Knitted Silk Scaffold for Effective Entheseal Chondrogenesis and Ligamentogenesis

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based on research carried out

under the supervision of

Prof. Bibhukalyan Prasad Nayak

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Assistant Professor

May 27, 2016

Supervisors' Certificate

This is to certify that the work presented in the dissertation entitled *Cross-linking of Knitted Silk Scaffold for Effective Entheseal Chondrogenesis and Ligamentogenesis* submitted by *Luv Kishore Srivastava,* Roll Number 214BM2031, is a record of original research carried out by him under my supervision and guidance in partial fulfillment of the requirements of the degree of *Master of Technology in Biotechnology*. Neither this dissertation nor any part of it has been submitted earlier for any degree or diploma to any institute or university in India or abroad.

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Bibhukalyan Prasad Nayak
Assistant Professor

Declaration of Originality

I, *Luv Kishore Srivastava*, Roll Number *214BM2031* hereby declare that this thesis entitled *Cross-linking of Knitted Silk Scaffold for Effective Entheseal Chondrogenesis and Ligamentogenesis* presents my original work carried out as a postgraduate student of NIT Rourkela and, to the best of my knowledge, contains no material previously published or written by another person, nor any material presented by me for the award of any degree or diploma of NIT Rourkela or any other institution. Any contribution made to this research by others, with whom I have worked at NIT Rourkela or elsewhere, is explicitly acknowledged in the thesis. Works of other authors cited in this thesis have been duly acknowledged under the section "Reference". I have also submitted my original research records to the scrutiny committee for evaluation of my thesis.

I am fully aware that in case of any non-compliance detected in future, the Senate of NIT Rourkela may withdraw the degree awarded to me on the basis of the present thesis.

May 27, 2016

NIT Rourkela

Luv Kishore Srivastava

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Abstract

The ligament is a connective tissue that bridges two bones and stabilizes the joints during motor activities. Anterior Cruciate Ligament (ACL) is one of the primary ligaments present in the knee connecting notch of the femur leads back into the femoral chondyle and fixes deep within tibia of the lower limb. ACL is the most commonly injured ligament among young and active individuals. Further, like other ligaments, ACL fails to heal optimally due to its low vascularity. The core insertional strength of ligaments is provided by a specialized organ called enthesis comprising of avascular fibrocartilage. Thus, if the current method of ligament tissue engineering would be integrated with pre-installation of enthesis at the bone-ligament interface, the original insertional strength can be envisaged in the tissue engineered construct. The current research project focuses on the fabrication of a silk based multi-compartmental scaffold, reinforced with natural polymers using a series of cross-linkers that can be used for ligament tissue engineering by enhancing the biocompatibility features. Briefly, the backbone of the scaffold was made by knitted silk followed by a coating of biopolymers like chitosan and gelatin for ligament and enthesial region respectively by the simple soaking method. To increase the coating yield and the stability of biopolymers on the knitted silk scaffolds, four different cross-linkers (i.e. araldite DY-T, PEG-dimethacrylate, glutaraldehyde and tyrosinase) were used and the outcomes were compared. These cross-linkers showed improvement in surface morphology, absorption, biodegradation and mechanical studies when compared with non-crosslinked scaffolds. TGA and FTIR spectroscopy were conducted for thermal and structural behavior analysis. MG-63 cells were used for biocompatibility of the scaffold using MTT assay. For both chitosan and gelatin coated scaffolds, tyrosinase was found to be the most suitable crosslinker on account of biocompatibility.

Keywords: ***Ligament; Enthesis; Silk; Chitosan; Gelatin; Cross-linking***

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Chapter 1

Introduction

1.1 Ligament

A ligament is a connective tissue, which joins a bone with an another bone. They are a dense bundle of fibres which connects one end of a bone with the other. The fibres of the ligament are flexible in nature and is the main reason why the joints are movable and not fixed. Besides this, even when the joint is at rest it brings stability to the joint and helps the bones to maintain their position. Ligaments are the tissues which are present with very high vascularity. Previously, ligaments were assumed to be inactive structures. However, studies have now proven it to be responsive to many local and systemic stimuli [1].

The ligament is a collagenous tissue, with collagen accounting for 75% of the dry weight. Other components include proteoglycans (1%), elastin, some proteins and a variety of glycoproteins, namely integrins, laminin and actin. Primary collagen that is present in ligaments is type I collagen (85%), and the rest is collagen types III, IV, V, XI, and XIV.

Tropocollagen molecule is the primary building [2] unit of a ligament. These molecules are arranged in the form of bundles to form fibres. The fibres are further organized into bundles called as fascicles, which combine to form a ligament. Fibroblasts are present in between the rows of these collagen fibres. The fibroblasts are involved in producing and maintaining the composition of the ECM. Besides this, they are capable of cell-to-cell communication. The proteoglycans present in the ECM store water and provide viscoelastic properties to the ligament. The viscoelastic nature allows the ligament to extend under tension and then regain its original shape when the tension is taken off.

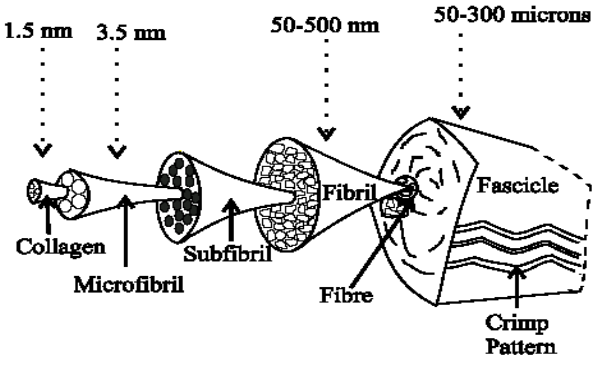


Figure 1.1 Ultrastructure of ligament [3]

There are different types of ligament tissues. The present study deals with knee ligaments. Knee ligaments are of four types:

i. Anterior cruciate ligament (ACL)
ii. Posterior cruciate ligament (PCL)
iii. Medial collateral ligament (MCL)
iv. Lateral collateral ligament (LCL)

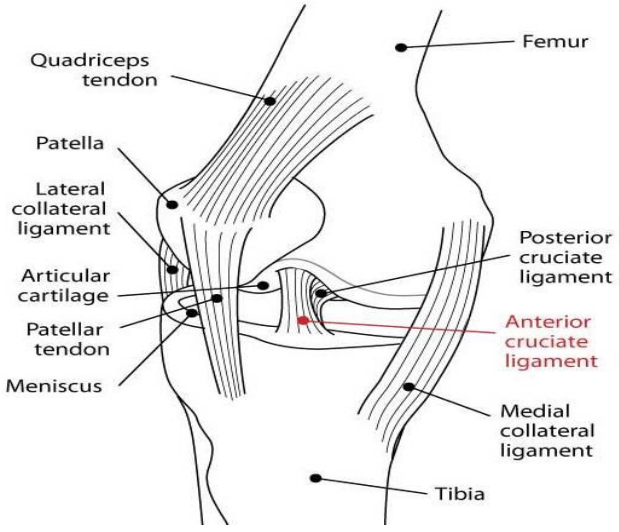


Figure 1.2 Ligaments in the knee [40]

Within a joint, ligaments are highly prone to injury and result in muscoskeletal joint pain and related problems. Depending on the causing factor, ligament injuries can be classified as intrinsic or extrinsic. Intrinsic ligament injuries are classified caused due to incorrect motion within the joint. Extrinsic ligament injuries occur due to external factors.

1.2 ACL

Anterior cruciate ligament (ACL) is the most important structure present in the knee. It is mainly responsible for resisting rotational and tibial translational loads. Due to its load bearing capacity, it is most commonly injured ligament. Due to lack of cellularity and vascularity it does not heal that easily [4]. The ACL mainly develops as a result of mesenchymal condensation during gestation period. ACL is composed of bands of dense fibrous connective tissues. It originates from a notch in femur and runs along the walls of the femoral condyle and finally gets inserted into the tibial plateau [5]. ACL consists of two

bundles namely posterolateral and anteromedial named on the site of insertion in tibial plateau.

Once, the ACL gets damaged then reconstruction is done by autografting. In autografting, the tissues required are taken from the patients' own body. Initially xenografts were also used but to factors like contamination and issues related to tissue rejection led to popularity of autograft

1.3 Enthesis

Connective tissues like ligaments and tendons are joined to bones by a specialized insertion site called as enthesis. This region of the torso is not very popular and not taken care of while treating tissue injuries. The major drawback in the present transplantation strategy is under development of enthesis which ultimately leads to tissue morbidity [6]. Since enthesis is responsible for bearing immense load, proper development of enthesis should be taken care of. On the basis of location inside the body enthesis are:

(i) Fibrocartilaginous enthesis – this enthesis is generally located at the junction between bone and ligament [7]. It mainly consists of fibroblast cells which are responsible for secreting the extracellular matrix (ECM).

(ii) Tendenous enthesis – This type of enthesis is most commonly found at the junction between bone and muscle. It is the most dense enthesis found inside the body which forms by the maturation of fibrocartilage tissue already present [8].

(iii) Calcified enthesis- as the name suggests, this type of enthesis is found after the ossification of fibrocartilage tissue and forms a continuity between two different tissues [7].

1.3.1 Structure of Enthesis

The design of enthesis is such that it facilitates even transmission of force. The enthesis may or may not be covered by a periosteum membrane. If the membrane is absent, then it leads to 'direct attachment' of the ligament with the bone, but if the periosteum membrane is present the, it is known as 'indirect attachment' [9]. Based on the structure, enthesis is basically of following two types: -

(i) Fibrous enthesis – in this type of enthesis an outer periosteum membrane is present to which the soft tissue gets attached. Since the soft tissue is attaching with the periosteum and not with the enthesis directly, this forms an 'indirect attachment'. This type of enthesis also lacks any cellular component [7].

(ii) Fibrocartilaginous enthesis- this is a much complex type of enthesis. Here, the periosteum is absent, and thus the attachment is of 'direct type'. Besides this, the fibrocartilaginous enthesis consists of cells which undergo chondrogenesis and leads to the formation of following zones:

- Ligament
- Uncalcified cartilage
- Calcified cartilage
- Bone

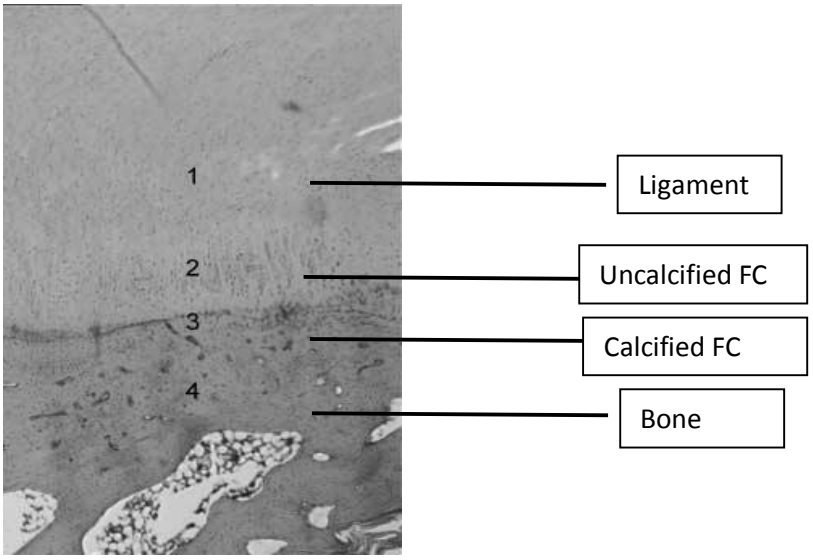


Figure 1.3 Structure of enthesis

These zones finally merge into one. Fibrocartilage entheses is the most common type of entheses inside body and therefore is the most demanded entheses for tissue engineering [7].

1.3.2 Injury at the enthesis

The structure of enthesis is such that, most of the stress is concentrated towards it. Though the stress-bearing capacity of enthesis is very high but in cases like excessive stress, accidents or bruises, it might get damaged. Most commonly, the enthesis gets damages in sportsperson which bear a lot of stress and after a certain limit the ligament gets damaged. Swelling of enthesis is called as enthesopathy [10]. Enthesopathy might occur due to diffuse idiopathic skeletal hyperostosis (DISH). The development of enthesis after injury is very important to regain full potential of the joint.

1.4 Biopolymers used in biomedical engineering

With an expanding population, the burden to improve the treatment of injured or diseased organ is also increasing. State-of-the-art technologies have been employed in treating or replacing an injured organ, but still the outcome remains suboptimal. Tissue engineering has been an essential tool as far as replacement of tissue in concerned. The credit goes to hard and soft tissue engineering, which now it is very much possible to reconstruct small sized tissue using patients own resources reducing the chances of tissue rejection [11].

Tissue engineering uses a biopolymer which is used to make a scaffold and finally the scaffold is seeded with cells for growth. These biopolymers have certain properties which make them suitable to be used as a scaffold. Some of these properties are [12]:

- Biocompatibility
- Biodegradability
- Non-toxic
- Non-immunogenic
- Adequate porosity

Different biopolymers are used for soft tissue or hard tissue engineering. It is not possible for a single biopolymer to possess all the above-mentioned properties. Thus, composites of different biopolymers are also used as a biomaterial for scaffold preparation.

1.4.1 Biopolymers in soft tissue engineering

The present strategies for soft tissue engineering depend on artificial implants which are restricted by many factors which includes tissue rupture and tissue resorption. Many natural biomaterials have been used for the scaffold preparation, and these biomaterials can be used singly or in composites on which different cells are grown. The table below indicates some common biomaterials used for scaffold preparation along with the cells which should be seeded on it [13][14][15][16].

Table 1.1: some polymers used in soft tissue engineering

Scaffold	Cells
Hyaluronic acid	Keratinocytes
Collagen gel	Fibroblasts, keratinocytes
Chitosan	Fibroblasts, keratinocytes
Gelatin	Fibroblasts, keratinocytes
PLA	Fibroblasts, keratinocytes
PEO	Fibroblasts, keratinocytes
poly (ester urethane) ureas	Keratinocytes
Poly lactic acid	Fibroblasts, keratinocytes

1.5 Cross-linking

Cross-linking is defined as the bonding between one polymer chain with the other. These bonds can be covalent or ionic with the polymer units being similar or dissimilar in nature. These polymeric units can be synthetic as in case of plastics and rubber. On the other hand, it can also be formed between natural biological sources like proteins and other biopolymers. The properties of the polymers get modified to a very large extent using cross-linking. They vastly modify properties like melting, boiling, stability, mechanical strength etc. Let us consider rubber for an example, the polyisoprene units of rubber gets cross-linked with each other forming a giant super molecule due to the help of small sulphur bridges. These bridges bring about an accumulation of polyisoprene units and this is the reason why tyres made up of rubber are not sticky or melt spontaneously. Besides this, the flexibility rubber possess is due to cross-linking [17].

Crosslinkers also have the potential to alter drugs, nucleic acids and solid surfaces. The same chemistry is applied to amino acid and nucleic acid surface modification and labeling. This area of chemistry is known as bio-conjugation and includes crosslinking, immobilization, surface modification, and labeling of biomolecules.

Recently cross-linking has discovered its wide application in biomedical sciences including wide applications in tissue engineering. A large number of biopolymers are used in tissue engineering but with a major drawback regarding their mechanical properties and stability in high humid and physiological environment. Cross-linking has grown to be one of the most important tool to overcome this major drawback. The cross-linking agents interconnects different molecules together resulting in an overall increase in its molecular weight and stability. A large number of crosslinkers and cross-linking techniques are present which mainly depends on the type of biopolymer being used. The aldehyde glutaraldehyde is the most popular cross-linking agent for tissue engineering purpose. The main reason for such wide use of glutaraldehyde is its ability to react the functional groups present in both carbohydrates and proteins. Simultaneously it provides a great enhancement in the mechanical properties and up to 8% glutaraldehyde was found to be non-toxic.

In this project, we plan to fabricate a scaffold, made by knitting silk fibres, for the fibrocartilage region of the ligament. This knitted silk scaffold will be subjected to surface modifications by grafting of bio-polymers like chitosan and gelatin on the knitted silk scaffold. It has been observed that grafting of these polymers is indigent on the silk fibres

present on the silk scaffold. So to improve the grafting of the bio-polymers, cross-linking is done. Cross-linking can be of many types: chemical, enzymatic and photo etc.

In chemical cross-linking, a chemical is used as a cross linker containing at least two functional groups, reactive in nature, facilitating formation of bonds between different chains of the polymer. In this project, epoxy resin, glutaraldehyde and PGA di-methacrylate will be used as chemical crosslinkers[18].

For cross-linking of chitosan, PEG-dimethacrylate is a good option on account of its solubility, but the most common cross linkers are dialdehydes, in particular, glutaraldehyde. These aldehydes directly take part in the reaction without the involvement of other reagents. Reaction between chitosan and glutaraldehyde is very well-documented. The aldehyde group of the glutaraldehyde forms a covalent imine bonds with chitosan through its amino group (Schiff reaction), because of the resonance stabilization involving adjacent double ethylenic bonds[19]. Chitosan is less reactive in acidic media because of its protonated amino group, whereas in basic or neutral media they easily react with aldehydes.

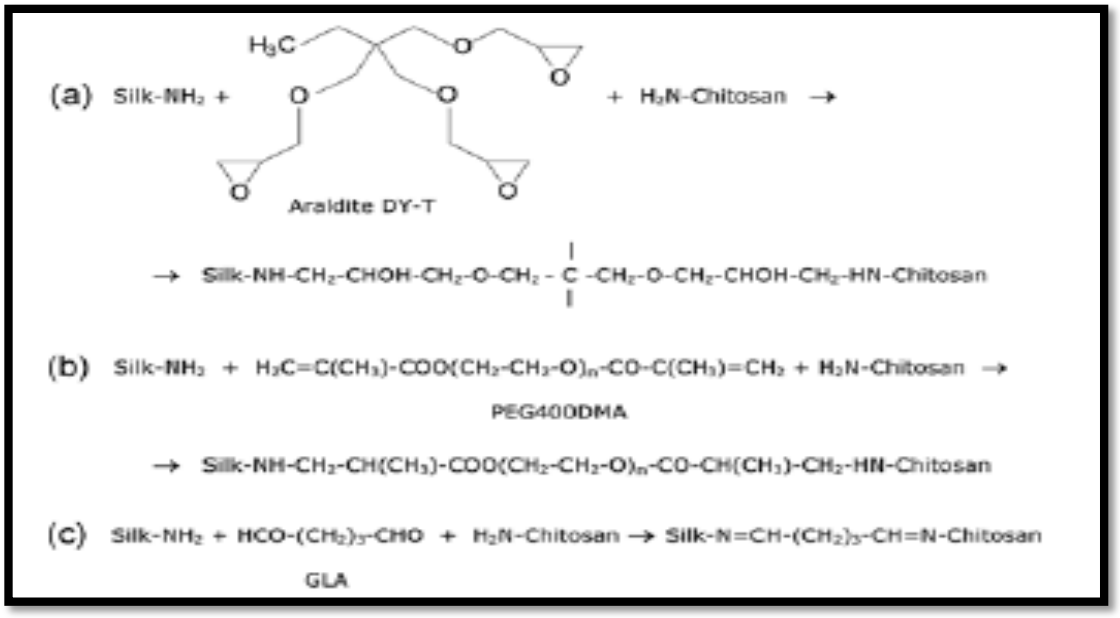


Figure 1.4 Reactions of Araldite DY-T, Glutaraldehyde and PEG di-methacrylate [18]

Cells require a conducive microenvironment to grow and toxicity can limit its use to a very larger extent. Hence, the need of a natural crosslinker gained importance and it was later found that enzymes proved to be very helpful for cross-linking activities without involving

the toxicity of chemicals. Tyrosinase (EC 1.14.18.1), is a polyphenol oxidase, it is responsible for the oxidation of tyrosine or 3,4-dihydroxyphenylalanine (DOPA) present in silk to *ortho* quinone (*o*-quinone). The hydroxyl group or amino group in chitosan reacts with *o*-Quinone through Michael addition or Maillard leading to formation of a stable covalent bond which is irreversible in nature [17, 18]. The overall reaction for this process is described as below:

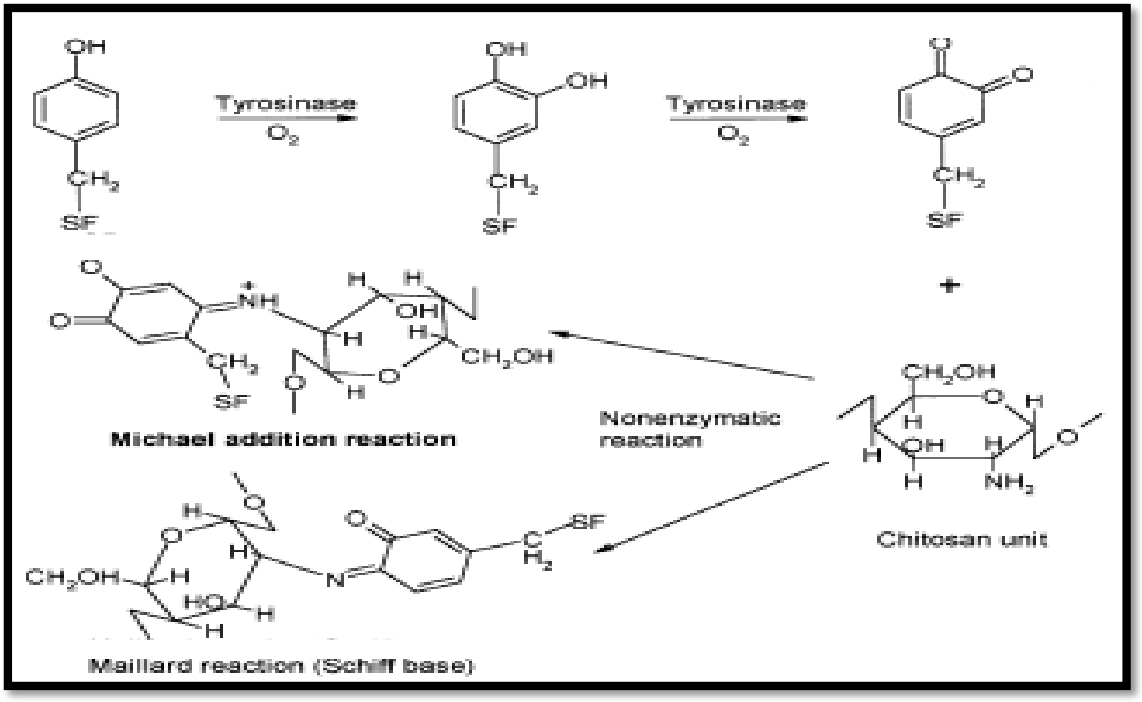


Figure1.5 Cross-linking reaction of tyrosinase on silk [20]

1.6 Objectives

Recent developments in tissue engineering have made it possible to reconstruct a tissue based on cytotherapy (application of live cells) and then seeding it on to a scaffold made up of suitable biomaterial. One of the major drawbacks regarding this is the stability and integration with the biopolymers. It is a well-known fact that natural sources will be less toxic to the overall mechanism of crosslinking due to its origin. The current research proposes the fabrication of scaffold using silk fibre. The silk fibres will be used to form the backbone of the scaffold by knitting on a knitting machine. This will be followed by coating of biopolymers like chitosan and gelatin. Since, the coating efficiency is not so high on the silk fibres, thus various crosslinkers will be used to enhance the coating efficiency and will

also provide a conducive environment for the cells to grow on. The various objectives of this research project are: -

- To show the significance of gelatin on knitted silk scaffold for ligamentogenesis,
- To show the significance of chitosan on knitted silk scaffold for entheseal chondrogenesis,
- To compare the efficacy of different crosslinkers on silk, and
- Physical and biological characterization of the scaffold

Chapter 2

Literature Review

2.1 Tissue engineering

Tissue engineering in a wider sense articulates the fundamental concepts of material science and molecular biology to find out live or biological substitute of failing organs or cells. Main approaches used in tissue engineering include development of polymer scaffolds which are carrier for the cells, cell growth and use of signaling molecules.

These artificially developed scaffolds provide suitable or more precisely natural environment to the cells to grow, proliferate and differentiate while signaling molecules signal the cell to regenerate new tissues. Scaffolds also help in release of signal molecules in controlled manner to regulate the cell growth properly.

Some of the desirable properties of scaffolds are

1. Non –toxic and biocompatible with the tissues
2. Should be biodegradable with desired rate of biodegradation
3. Should not evoke immunological response in the body
4. Should have proper mechanical strength and porosity for releasing the desired substance in a controlled manner.

Being avascular in nature articular cartilage are difficult to repair spontaneously of its own [22]. Recently tissue engineering concepts have been used extensively as well as are in due process of research to overcome this difficulty. The field thus developed is known as cartilage tissue engineering which utilizes approaches which are cell based in nature that is concepts of molecular biology are used [23][24].

A variety of biomaterials are used as cell carriers [25]. Some notable examples are:

1. Scaffold matrices of type I and type II collagen based biomaterials.
2. Different types of simple gels
3. Collagen-alginate based gels

The cell material being used as cell carrier should act similar to the naturally occurring naturally occurring material in the articular cartilage for instance ECM (cartilage specific extracellular matrix) and GAGs (Glycosaminoglycan) promote chondrogenesis in vivo and in vitro [26].

2.2 Ligament tissue engineering

Ligament is the connecting link between two bones across a joint. It helps in proper alignment of the bones to prevent any dislocation. The most vulnerable ligament to fracture is that of knee joint. The most commonly injured ligament in the knee is ACL due to the immense load it bears.

Ligament tissue engineering is a new substitute to surgical grafting. In tissue engineering biomaterial scaffolds are fabricated which are used as platforms to grow and proliferate cells in vitro. The tissues thus produced are used to repair or replace injured tissue. Earlier this technique was popular for repairing skin (wound healing), bone, cartilage etc. But in the recent times, ligament tissue engineering has grown to be a promising field to produce neo-ligaments. These engineered tissue grafts are very much successful in long run as they do not lose strength over the time unlike traditional grafts. Due to their biocompatibility they can be incorporated in the host tissue very well without invoking any immunological response. By selecting proper biomaterial with precise pore size ligament fibroblasts can be successfully grown and can replace traditional grafts.

2.3 Interface Tissue Engineering

During the due course of research, it has been seen that the tissue grafts grew well in in-vitro conditions but when scaffold was transferred to the body (in vivo) it could not integrate itself in the bones which were present inside the knee. The principle reasons behind the failed experiments were observed to be following: -

- Scaffold was loosely fixed at the site of injury
- Early biodegradation of scaffold material
- Non compatibility of the graft at molecular and cellular level.

Further research showed that it was the presence of Enthesis (fibro cartilaginous cell at the junction between ligament and bone) in natural conditions which was not present in

fabricated ligaments. Further advancements in the tissue engineering was done by including enthesis as a part of the scaffold besides ligament and bone.

2.4 Approaches of interface tissue engineering

In the light of failed experiments scientists shifted their approach to micro and nano structure of the interface and introduce advanced scaffolds that mimic typical cellularity and complex biochemical properties through co-cultures and growth factor gradients.

The approaches can be categorized as:

- Scaffold-based strategies: multi-phased scaffolds;
- Cell-based strategies: stem cells and co-cultured cells;
- Growth factors and gene therapy;
- Mechanical loading in bioreactors.

2.5 Chitosan

In recent times chitosan has grown to be a very popular biopolymer in tissue engineering. The fundamental reason for this is the remarkable biological properties chitosan exhibits. Chitosan expresses the properties like anti-tumor, immune-enhancing, nontoxic, highly biocompatible and biodegradability which makes it such a popular biopolymer for biomedical and other uses. Chitosan is procured by deacetylating chitin obtained from shells of marine crustaceans. Chitosan is a linear hetero-polysaccharide consisting of glucosamine (GlcN) and N-acetyl glucosamine (GlcNAc) joined together by β-(1-4) linkage. The molecular weight of chitosan may vary from 50 to 100kDa and the degree of deacetylation from 25% to over 95% and depends on the processing and origin of chitosan [22]. The degree of deacetylation has a direct impact on the biodegradability of chitosan as more the deacetylation will be it will be tough for enzymes like lysozyme to degrade it into oligosaccharides [27]. Chitosan has a stable crystalline structure and due to this it is not soluble in aqueous solution or at pH close to 7. But at low pH the amine group of chitosan gets protonated and it becomes soluble at pH close to 5 [28]. Besides enhancing the solubility, the protonated positively charged amine group binds many negatively charged molecules like cytokines and growth factors [29]. The protonated amine group also binds with the anions present on the walls of microbes which ultimately results in rupturing the cell membrane and the contents leak out.

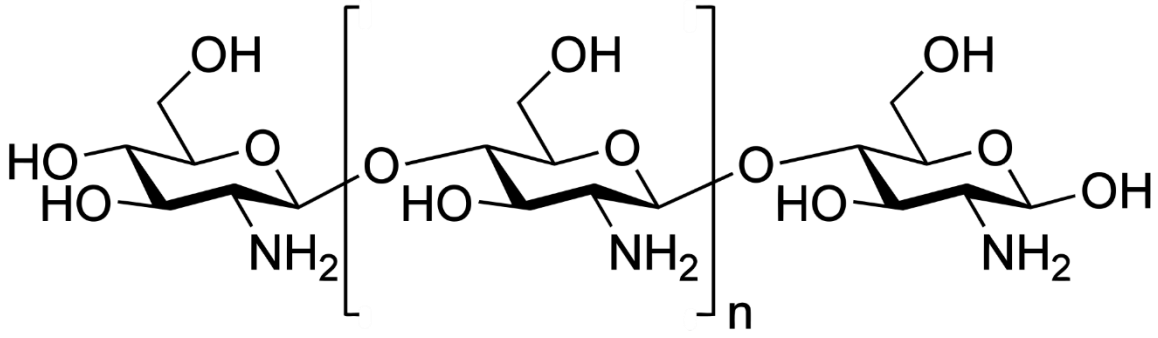


Figure 2.1 Structure of chitosan

In tissue engineering

Chitosan has a property by which it can be moulded into desired forms [29]. It also has a tendency to form a porous scaffold after lyophilizing which is a boon for tissue engineering purpose. Chitosan mimics some of the properties of hyaluronic acids and GAGs which make them a popular material in tissue engineering. The chitosan contains N-acetyl glucosamine moiety which is structurally similar to the features present in GAGs. GAG properties include a large number of specific interactions with receptors, growth factors, and proteins involved in adhesion. Because of this similarity between GAG and chitosan, it can be safe to say that chitosan might show similar bioactivities. The implants having chitosan are found to evoke very less foreign body response. No major case of immunological response has been reported so far making chitosan principle material in tissue engineering.

One of the most promising feature of chitosan is that it can be molded into various pore sizes of choice to be used in transplantation of cells and regeneration of tissue. Chitosan can be made porous by lyophilizing chitosan-acetic acid solution in molds of suitable size [30]. By varying freezing rate pore size can be controlled.

2.6 Silk

Silk is a fibrous protein. Many species of silkworms and spiders spun silk [31]. Silkworm silk contain an immunogenic substance known as sericin, and hence, it has to be degummed before using it as a biomaterial. The silk can be processed by combining it with other materials like gelatin etc. to make it more qualified candidate as a biomaterial. On the other hand, spider silk does not contain sericin and thus can be used in natural fibre form.

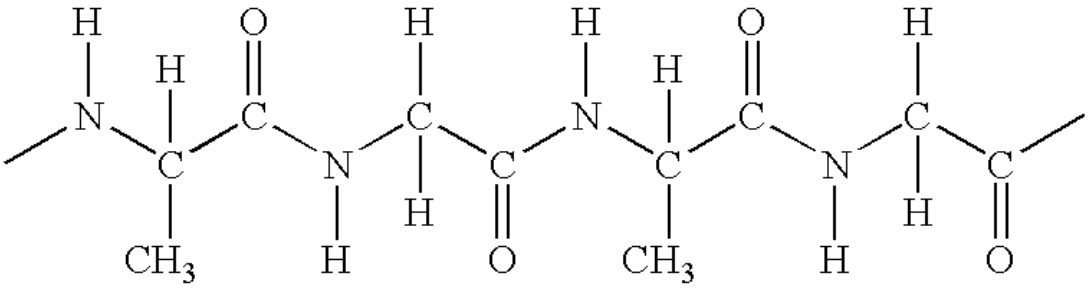


Figure 2.2 Structure of ligament

In tissue engineering

Silk fibres are glued into strands via sericin. Sericin is an immunogenic substance thus has to be removed from the fibres [32]. Although degumming is done to get rid of sericin but this processing results in altering the mechanical properties of the silk fibres [33]. Principle method used for processing via production of fibroin solution, in which degummed silk is dissolved which causes significant loss in natural mechanical properties of silk strands. After this silk can be coated on to other materials or can be reproduced in the form of thin films as per the convenience [34]. Silk thus obtained can be spun into porous scaffolds by using different techniques like freeze drying, salt leaching, gas foaming and many other phase separation methods.

Silk can be used in simpler form or it can also be amalgamated with other substances to make it more suitable biomaterial. Silk can be used in the form of hydrogels by combining it with gelatin or it can also be drawn in to sheets known as nano-hydroxyapatite sheets by combining it with hydroxyapatite [35].

Dermal fibroblasts proliferate and differentiate on silk scaffolds without evoking any inflammatory response. Oral keratin cells have also been reported to proliferate effectively on fibroin meshes. It has also been reported that fibroin-alginate sponges enhance the rate of wound healing as compared to other clinically used materials. A combination of fibroin - chitosan meshes has been used in the healing of ventral hernias [36].

Porous Fibroin scaffold have been used in cartilage tissue engineering by many researchers. These scaffolds are often developed by the process of salt leeching. Chondrogenesis studies have been done using these scaffolds using human chondrocytes and mesenchymal stem cells. Successful production of cartilage cells on the surface of silk fibres have been reported.

However mechanical properties of this fabricated cartilage were not in sync with the naturally occurring cartilage. Studies suggests that optimization of media is required to modify mechanical properties of the engineered silk. Scaffolds developed from regenerated spidroin with specific porosity have shown promising results in chondrocyte attachment and proliferation [37].

B. mori silk (after degumming) is spun into yarns and is a potential candidate in ligament tissue engineering. The surface morphology of the yarn can be altered by further processing to make it viable for the proliferation of ligament cells. It has been reported that anterior cruciate ligament fibroblasts [38] adhere and migrate on silk yarns expressing various specific markers of the ligament, although cells did not migrate to the centre of individual cords. Silk fibres or yarns can also be coated with the gelatin to restore the mechanical strength lost by degumming process and further it can be used as a promising biomaterial.

2.7 Gelatin

Gelatin is obtained by hydrolysis of collagen and have properties almost similar to that of collagen [39]. Gelatin has emerged as a material of choice for fabricating scaffolds as it is a denatured polymer therefore problems related to immunogenic responses as well as pathogen transmission are subsided.

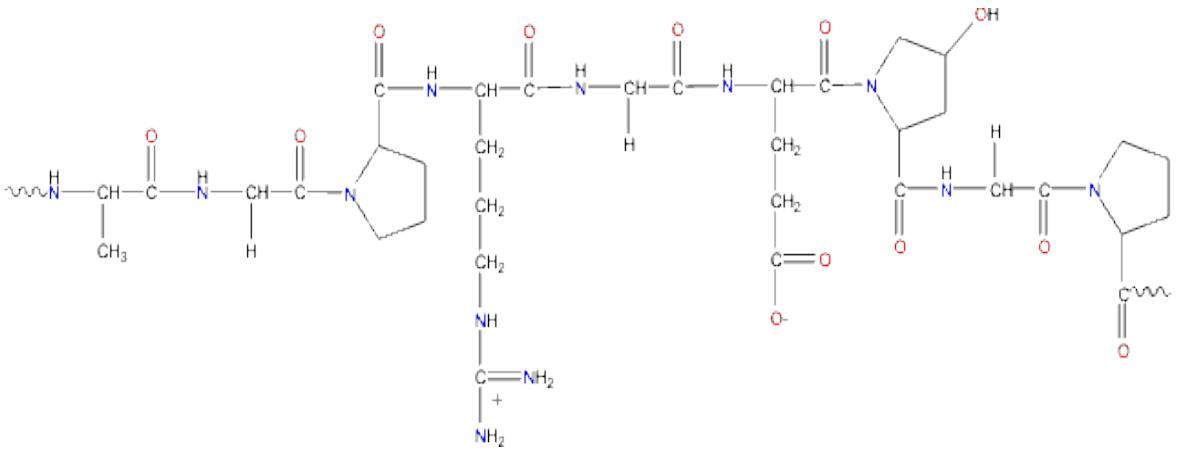


Figure 2.3 Structure of gelatin

In tissue engineering

Gelatin nano fibrils are generally developed by the method of electrospinning. In this method gelatin is first dissolved in some specific solvents like 1,1,1,3,3,3 hexafuoro-2-propanol and 2,2,2-trifluroethanol and then it is fabricated into nano fibrils. Electrospinning produce 2-D sheets which cannot be used as such in tissue engineering as for attachment and proliferation of cells specific pore size and architecture is required. This feature is not present in electros punned 2-D sheets. But when gelatin is amalgamated with HAP (hydroxyapatite) into composite scaffolds results in bringing together favorable properties of both HAP and gelatin. This combination contains nano size pores and desired architecture similar to that of natural ECM (extracellular matrix) which can be used for cell proliferation.

Porous gelatin scaffolds can be produced by swelling, cross-linking and lyophilization. The porogen method is used to create the pores of specific size. In porogen method phase separation is done using organic solvents.

To produce porous scaffolds using gelatin porogen method can be used. In this method, water is used as solvent followed by lyophilization results in porous scaffolds. The pore size and pore structure can be regulated by varying the rate of freezing. This technique has been successfully used in the fabrication of alginate and collagen sponges.

Chapter 3

3. Materials and Methods

3.1 Fabrication of Scaffold

3.1.1 Formation of silk yarn

The silk procured was in the form of thin fibroins. To make it knittable, the thin silk fibroins were gelled into a thick 6 ply fibre. This was done by applying polymers like chitosan and gelatin to a bundle of 6 thin fibroins respectively. After applying the biopolymer, the fibres were left to air dry and finally a thick knittable fibre was obtained. Now, the fibres were ready to be knitted on a knitting machine.

Figure 3.1 Air drying of gelled silk fibres

3.1.2 Knitting of silk

The knitted silk meshes were made by using a Brother Knitting Machine. The size covered by 6 alternate needles was considered to be optimum. Pore size was set to minimum. Firstly, the silk yarn was loaded into the K-carriage by keeping it on the left end. 6 alternate needles were placed in the 'out' position. The K-carriage was pulled to the right in the 'working' position. Once placed in the 'working' position, the remaining needles are brought forward to join with the previously alternate needles. The 'claw' weights were placed at the needles to provide the downward weight. The K-carriage is now moved left and right till a mesh of desired width is obtained. The knitted silk mesh is then cut free from the K-carriage and the needles.

Figure 3.2 Brother knitting machine

Figure 3.3 Knitted silk scaffold

3.2 Preparation of solutions

3.2.1 Chitosan solution

Chitosan from Himedia (Degree of Deacetylation >90%) was used for the preparation of chitosan solution. 2% (wt/vol) chitosan solution was made by 2gms of chitosan powder in 100ml 2% (vol/vol) acetic acid. For a homogenous solution, the mixture was kept overnight on a magnetic stirrer at 300 rpm.

Figure 3.4 Chitosan solution

3.2.2 Gelatin solution

Gelatin from HiMedia was used for the preparation of gelatin solution. 4% (wt/vol) gelatin solution was made by dissolving 4gms of gelatin in 100ml of distilled water. Since gelatin is sparingly soluble in water at room temperature, it was dissolved at 90˚C.

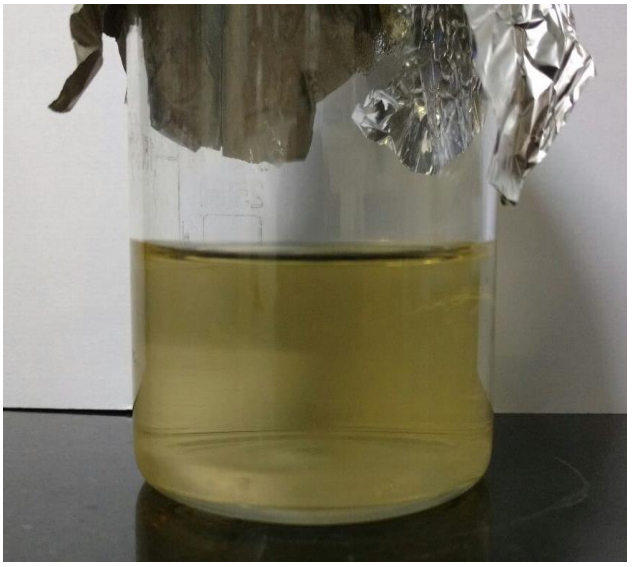


Figure 3.5 Gelatin solution

3.2.3 PBS solution

Phosphate Buffered Saline (PBS) is a very important buffer used in various experiments. Its similarity with body fluids makes it popular to simulate those experiments which will finally end up *in-vivo*. To simulate the fluid, present inside the body PBS proves to be a very good tool. 1L of 1X PBS solution can be prepared by taking. 8gms of Sodium chloride (NaCl) followed by 0.2g of potassium chloride (KCl) and dissolving it in 1L of distilled water. After this,1.44g of Na_2HPO_4 and then KH_2PO_4 was added. Since, this buffer simulates body fluid, the pH is adjusted to 7.4 at 25˚C.

3.3 Coating of Biopolymers on knitted silk

3.3.1 Without crosslinkers

The grating operation was done by soaking method. In a polypropylene tube, the knitted silk scaffolds, tied on a glass slide, were soaked in chitosan or gelatin solution respectively. The setup was placed in a water bath at 60˚C for 2 hours. After 2 hours, the soaked scaffolds were dried in hot air oven at 75˚C for 30 minutes completing the process of grafting.

3.3.2 With crosslinkers

Various crosslinkers were used to enhance the coating of biopolymers on the knitted silk scaffold. The procedure used for coating was simple soaking method.

a) Using Araldite as crosslinker

The crosslinker araldite was added to chitosan and gelatin solution respectively in the ratio two parts of the solution to one part of araldite. The knitted silk scaffolds were soaked in this mixture at 50˚C for 2 hours. After the soaking process was complete, the scaffolds were cleaned thoroughly with phosphate buffered saline to remove physically adhered biopolymer [18].

b) Using PEG Dimethacrylate as crosslinker

The crosslinker PEG dimethacrylate was added to chitosan and gelatin solution respectively in the ratio two parts of the solution to one part of crosslinker. The knitted silk scaffolds were soaked in this mixture at 50˚C for 2 hours. After the soaking process was complete, the cross-linked scaffolds were cleaned thoroughly with phosphate buffered saline to remove physically adhered biopolymer [18].

c) Using glutaraldehyde as crosslinker

1% glutaraldehyde solution was prepared and added to chitosan and gelatin solution respectively in the ratio two parts of the solution to one part of crosslinker. The knitted silk scaffolds were soaked in this mixture at 50˚C for 2 hours. After the soaking process was complete, the cross-linked scaffolds were cleaned thoroughly with phosphate buffered saline to remove physically adhered biopolymer [18].

d) Using tyrosinase as crosslinker

The activity of tyrosinase was adjusted to 100 U/ml. This was added to chitosan and gelatin solution respectively. The knitted silk scaffolds were soaked in this mixture at 50˚C for 2 hours. After the soaking process was complete, the cross-linked scaffolds were cleaned thoroughly with phosphate buffered saline to remove physically adhered biopolymer [20].

3.3.3 Weight gain and Coat yield

The amount of polymer coated was calculated by weight gain percentage and coat yield %. These equations are very useful in the comparing the efficiency of grafting for different crosslinkers. The crosslinker with a higher graft yield is supposed to be superior over the other crosslinkers for the process of grafting.

$$weight\ gain\ (\%) = \frac{W - Wo}{Wo}\ X\ 100$$

$$coat\ yield\ (\%) = \frac{W - Wo}{Ws}\ X\ 100$$

Here, Wo is the weight of knitted silk; W is the weight of grafted silk and W_s is the weight of chitosan or chitosan and crosslinker together

3.4 Characterization Studies

3.4.1 Field Emission Scanning Electron Microscopy (FE-SEM) to study morphology

The morphology of the grafted silk scaffold was studied in FEI-Nova NanoSEM 450 field emission scanning electron microscope with the help of gold coating. The scaffold, cut into small pieces, were loaded onto the sample holder of the device. The scaffold was coated with gold for 5 minutes and then observed under the microscope. The electric field of FE-

SEM was 5KV and was observed at a magnification of 500X and1000X.

Figure 3.6 Nova NanoSEM 450

3.4.2 Swelling study

The water absorption or swelling studies were carried out for both non-crosslinked as well as crosslinked scaffolds. The weight of dry scaffolds was noted down followed by soaking them in phosphate buffered saline. The weight of the soaked scaffolds was noted at 60 minutes from the time of soaking. The same process was repeated again and the weight of the scaffold was recorded after every 60 minutes until no change in the weight was observed. This was the saturation point of the scaffold beyond which it could not take up any more liquid. To calculate the percentage of water absorbed, following formula was used:

$$\%\ absorption = \frac{Wn - Wo}{Wo}\ X\ 100$$

The absorption of the scaffolds depends on parameters like the affinity between the scaffold and water molecules along with the dimension of the scaffolds. For true readings, the scaffolds were kept almost equal in size.

3.4.3 Biodegradation study

Biodegradation studies were done for each type of scaffold. The initial dry weight of the

scaffolds (denoted as W_0) was recorded and then immersed in PBS at pH 7 in a 12 well plate. The setup was incubated at 37˚C for the degradation to occur. The degradation study was carried out at 3, 7, 14, 21 and 30 days respectively. On these days, the scaffold was taken out and then dried in a hot air oven at 75˚C for 30 minutes followed by recording its weight (denoted as W). The loss in weight is a very useful parameter to determine the weight loss as it directly gives the information of the amount of scaffold degraded. Loss in weight can be calculated using the following formula:

$$\%\ weight\ loss = \frac{Wo - W}{Wo}\ X\ 100$$

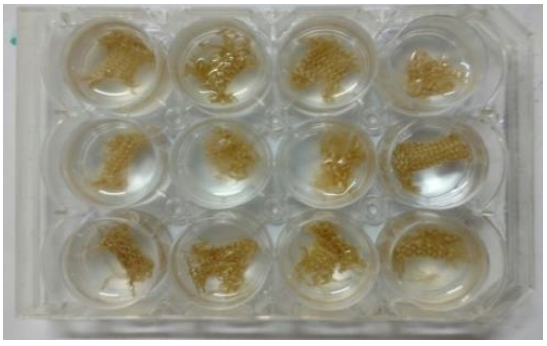


Figure 3.7 Biodegradation setup

3.4.4 Thermogravimetric (TGA) analysis

TGA was done to determine the effect of temperature on biodegradation of different scaffolds. It was done for the temperature range from 30˚C - 400˚C at the rate of 10K per minute in an argon atmosphere.

3.4.5 Differential scanning calorimetry (DSC) analysis

DSC was done to determine the glass transition temperature of the scaffolds. It was done prior to coating and after coating. Then the results were compared to observe the change in behavior of the scaffolds. It was done for the temperature range from 30˚C - 400˚C at the rate of 10 K per minute in an argon atmosphere

3.4.6 FTIR studies

The FTIR studies were done between the range 400 cm^{-1} to 4000cm^{-1} to detect the functional groups present in the scaffold. This gives an idea whether the functional groups of the cross-linkers are present in the scaffold or washed away.

The FTIR analysis was done to determine the functional groups present in the scaffold which tells about the composition of the scaffold. This is necessary to determine whether the biopolymer and the crosslinker are present on the scaffold or washed off. The FTIR analysis here is done to identify the biopolymers chitosan and gelatin. Besides this, it was done to determine the presence of the crosslinkers like araldite, PEG-D, glutaraldehyde, and tyrosinase.

3.5 Cell study

3.5.1 Cell culture

A standard protocol was followed for the culture of the MG-63 cell line. The cells were cultured in a T-75 culture flask with DMEM growth media supplemented with 5% foetal bovine serum. From time to time the flasks were observed for confluency. When the confluency reached 80%, the cells were subcultured in a new flask. For subculturing, firstly the media was taken out from the flask followed by washing three times with PBS. Now, trypsin solution was added to the flask to remove the adhered cells from the bottom of the flask. This was kept in a CO_2 incubator at 37˚C for 2 minutes. To stop the action of trypsin, fresh media containing serum was added to the flask. The cells, now present in the suspension, are subjected to centrifugation at 800 rpm for a period of 5 minutes. Now, the pellet formed was re-suspended and distributed into new flasks in the ratio 1:4.

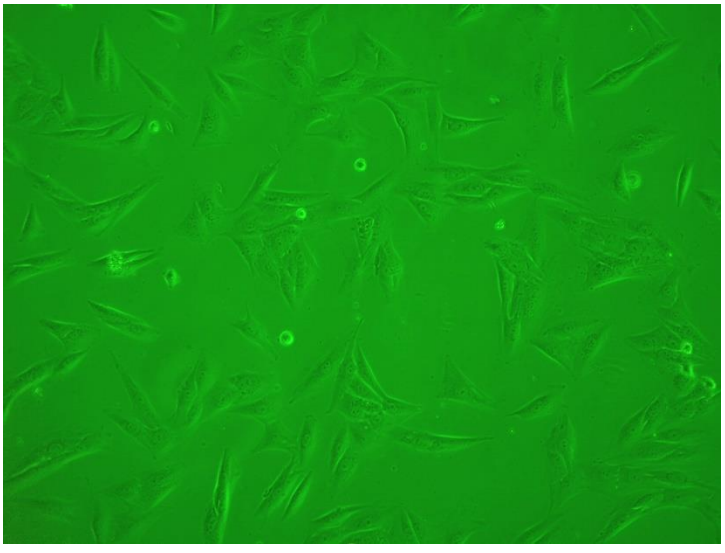


Figure 3.8 MG-63 cells

3.5.2 Scaffold sterilization

The scaffolds were sterilized before cell seeding. They were placed in petri-dishes and then was kept in a biosafety cabinet. A separate petri-dish containing 37% formaldehyde solution was also kept inside the cabinet. The fumes of formaldehyde start penetrating the scaffolds and decontaminates them. To remove the remaining formaldehyde, the coated knitted scaffolds were washed three times with autoclaved phosphate buffered saline. The scaffold was now exposed to UV light for 10 minutes. The scaffolds were dried and used for cell seeding.

3.5.3 Cell seeding

Cell seeding was done in a 24 well plate following the static seeding method. The MG-63 cells were seeded onto the scaffolds at a cell density of 1 X 10^4 cells/ml. After 30 minutes of cell seeding, DMEM media containing 5% FBS was added.

3.5.4 Cell viability assay

MTT assay was done using MTT assay kit from cell culture. The media was supplemented with MTT solution at a concentration of 4µl/100µl and kept in an incubator at 37˚C for 3 hours. Now, the formazan crystals were formed by the reaction between cytochrome oxidase and MTT and were dissolved using DMSO. This set up was kept at 37˚C for 25 minutes so that complete dissolution of formazan occur. Using a spectrophotometer, the optical density of the resulting solution at 590 nm.

Chapter 4

4. Results & Discussion

4.1 Weight gain and Graft yield

4.1.1 Coating without crosslinker

From Figure 4.1(a) and (b), weight gains and graft yield of gelatin coated scaffold were found to be more than that of chitosan coated scaffold. For gelatin and chitosan, the weight gain (%) was found to be 12.90% and 4.61% whereas the graft yield (%) was found to be 10% and 3.75% respectively.

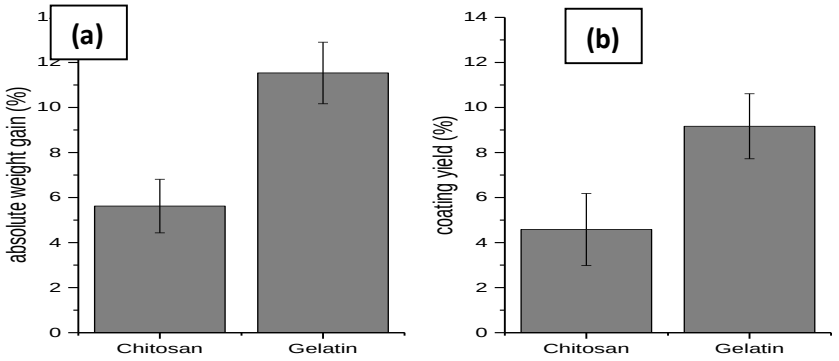


Figure 4.2 (a) Weight gain (%) (b) Coating yield (%)

4.1.2 Coating using crosslinker

Crosslinker Araldite DY-T was found to have maximum coating efficiency for chitosan coated scaffolds (Figure 4.2 a & 4.3 a) whereas for gelatin coated scaffolds (Figure 4.2 b & 4.3 b), PEG-dimethacrylate was found to be most efficient.

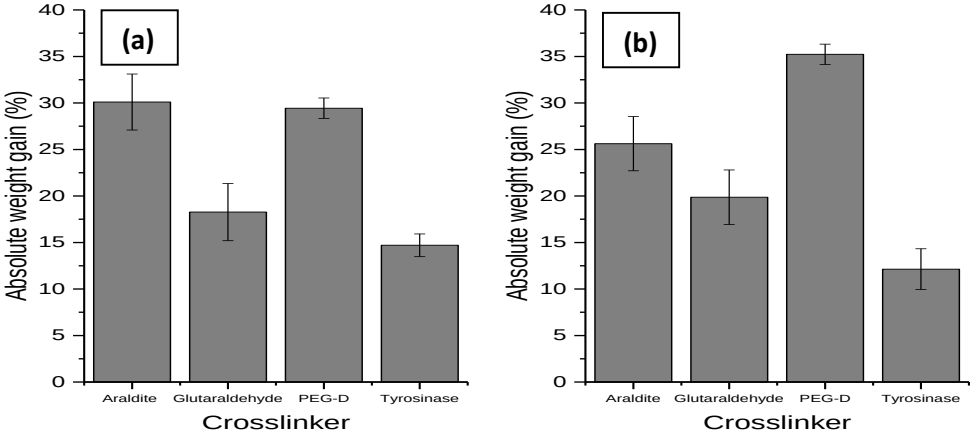


Figure 4.3 Weight gain (%): (a) Chitosan coated scaffold, and (b) Gelatin coated scaffold

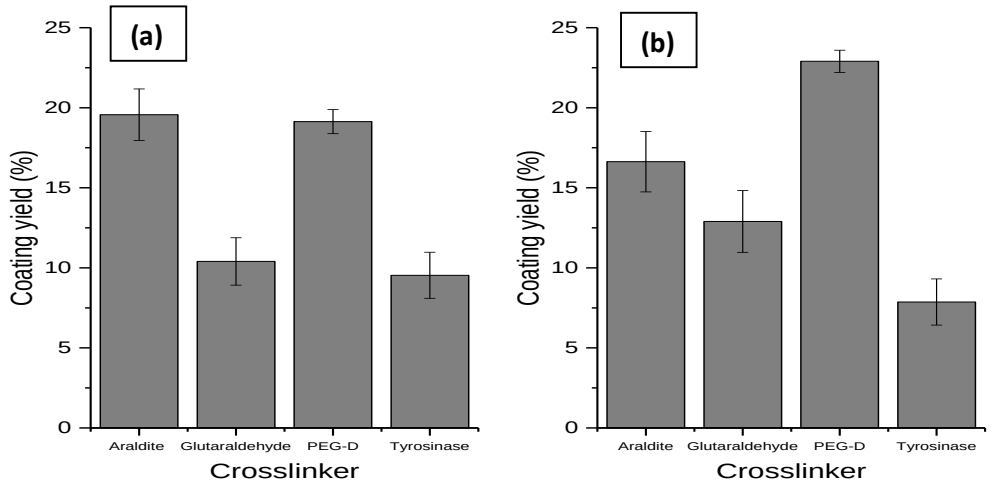


Figure 4.4 coating yield (%): (a) Chitosan coated scaffold, and (b) Gelatin coated scaffold

4.2 Characterization Studies

4.2.1 Water Absorption

A) Without cross-linking

From Figure 4.4 and 4.5 it can be seen that the water absorbing capacity of chitosan was more than that of gelatin and simple non-coated silk.

	0	60	120	180	240	300	360	420	480
Non-coated Scaffold	**0.064**	**0.141**	**0.157**	**0.164**	**0.170**	**0.171**	**0.171**	**0.172**	**0.172**
Chitosan coated scaffold	**0.062**	**0.155**	**0.186**	**0.197**	**0.211**	**0.235**	**0.248**	**0.249**	**0.250**
Gelatin coated scaffold	**0.065**	**0.137**	**0.144**	**0.147**	**0.150**	**0.152**	**0.155**	**0.156**	**0.156**

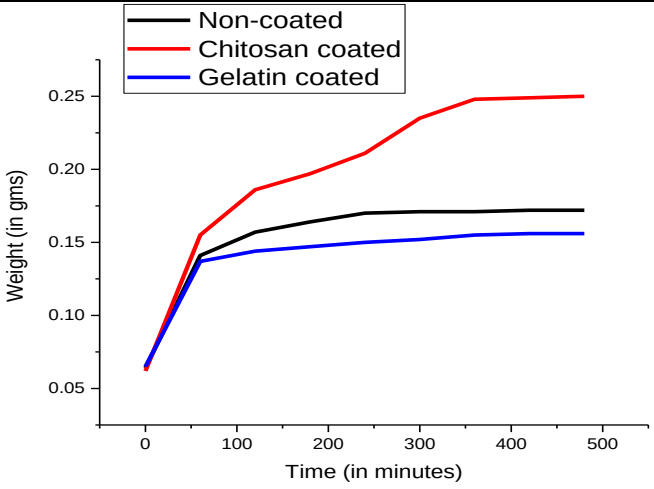


Figure 4.5 Water absorption

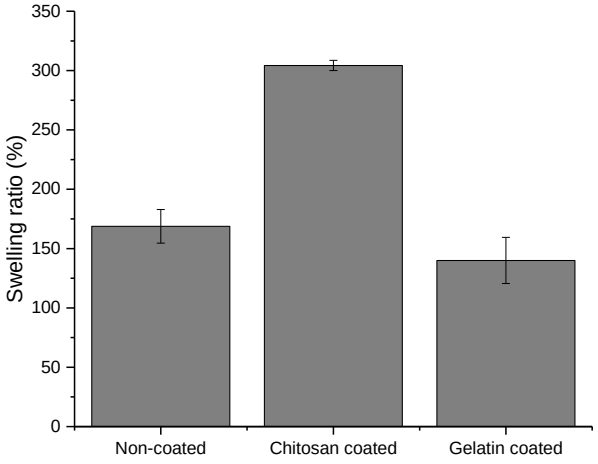


Figure 4.6 Water absorption comparison

B) After cross-linking

	0	**60**	**120**	**180**	**240**	**300**	**360**	**420**	**480**
Scaffold coated with chitosan using araldite as crosslinker	0.053	0.126	0.128	0.136	0.145	0.153	0.168	0.170	0.172
Scaffold coated with chitosan using PEG-dimethacrylate as crosslinker	0.052	0.111	0.134	0.144	0.152	0.158	0.162	0.169	0.170
Scaffold coated with chitosan using glutaraldehyde as crosslinker	0.051	0.133	0.139	0.145	0.151	0.162	0.179	0.186	0.187
Scaffold coated with chitosan using tyrosinase as crosslinker	0.052	0.139	0.145	0.152	0.159	0.166	0.189	0.210	0.221
Scaffold coated with gelatin using araldite as crosslinker	0.052	0.140	0.154	0.156	0.162	0.169	0.177	0.180	0.181
Scaffold coated with gelatin using PEG-dimethacrylate as crosslinker	0.055	0.101	0.118	0.121	0.127	0.132	0.134	0.134	0.135
Scaffold coated with gelatin using glutaraldehyde as crosslinker	0.052	0.112	0.124	0.145	0.150	0.157	0.163	0.165	0.165
Scaffold coated with gelatin using tyrosinase as crosslinker	0.043	0.141	0.145	0.155	0.155	0.156	0.156	0.157	0.158

For both chitosan scaffolds (4.6 a and 4.7 a) and gelatin scaffolds (4.6 b and 4.7 b) tyroinase increased water absorbtion capacity whereas other crosslinkers reduced it.

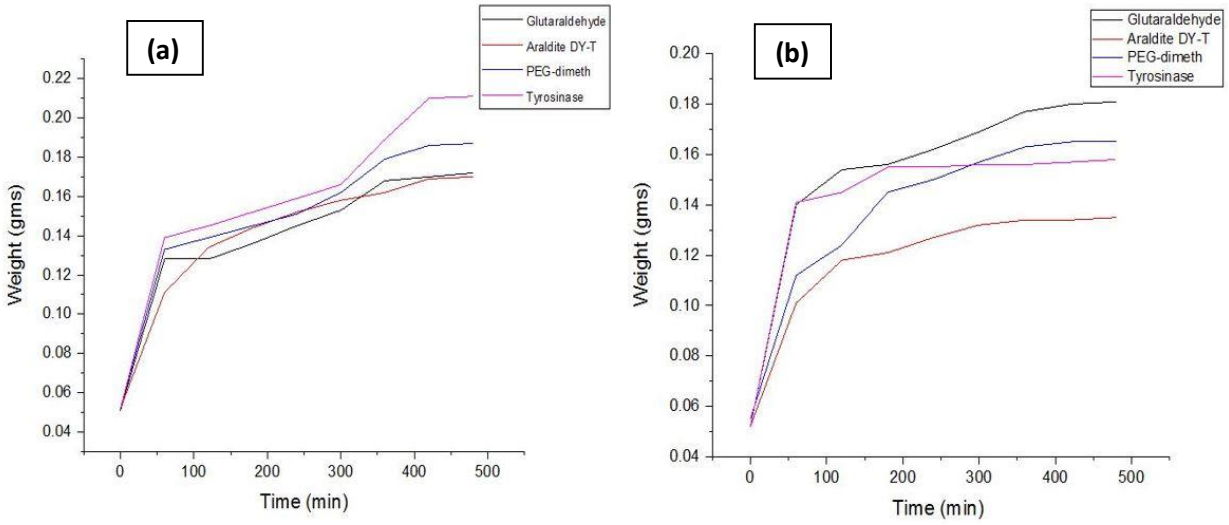


Figure 4.7 Weight gain for (a)chitosan and (b)gelatin coated scaffolds

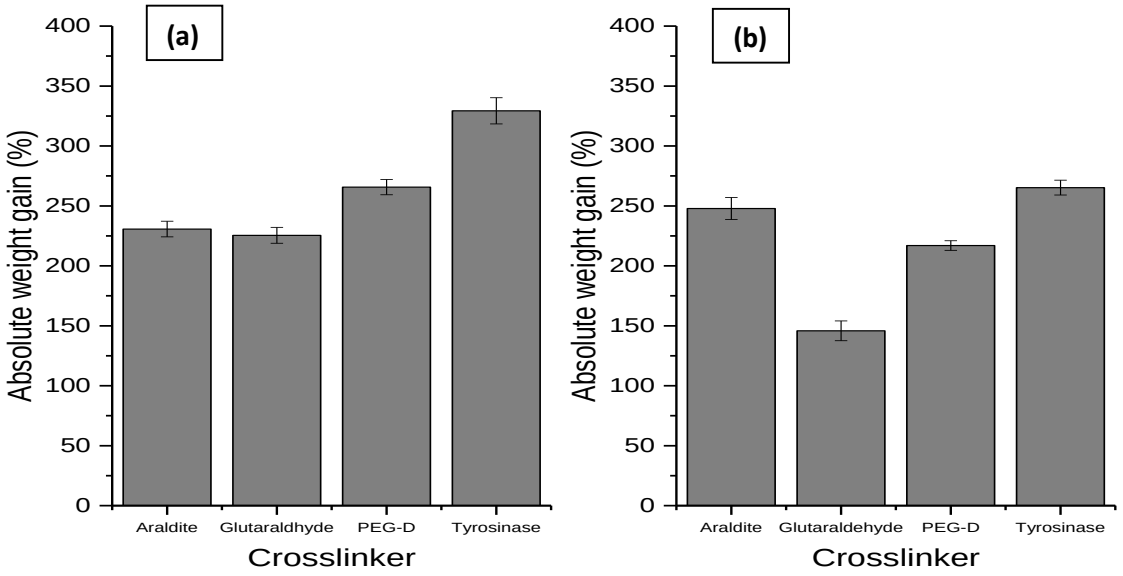


Figure 4.8 Swelling ratio of (a) chitosan and (b) gelatin coated scaffolds

4.2.2 FE-SEM analysis

Knitting process involves a knitting machine which might damage the silk fibres. It is imperative to maintain the integrity of the silk fibres which otherwise will result in reduced

tensile strength. FE-SEM image of the scaffold at 500X (Fig.18) clearly shows that during the knitting process no damage has been done to the silk fibres.

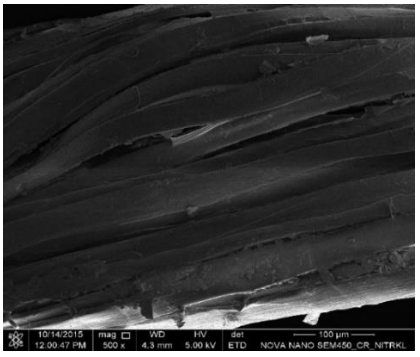


Figure 4.9 500X magnification of scaffold

a) Coated scaffold without crosslinker

Besides this, a general idea can be perceived by visualizing the 500X magnified FE-SEM images of non-coated and coated scaffold. The images clearly show that non-coated scaffold has the smoothest surface (Fig.19 a), whereas gelatin coated scaffold had the roughest (Fig.19 b) indicating that grafting of chitosan was more as compared with that of chitosan coated scaffold (19 c).

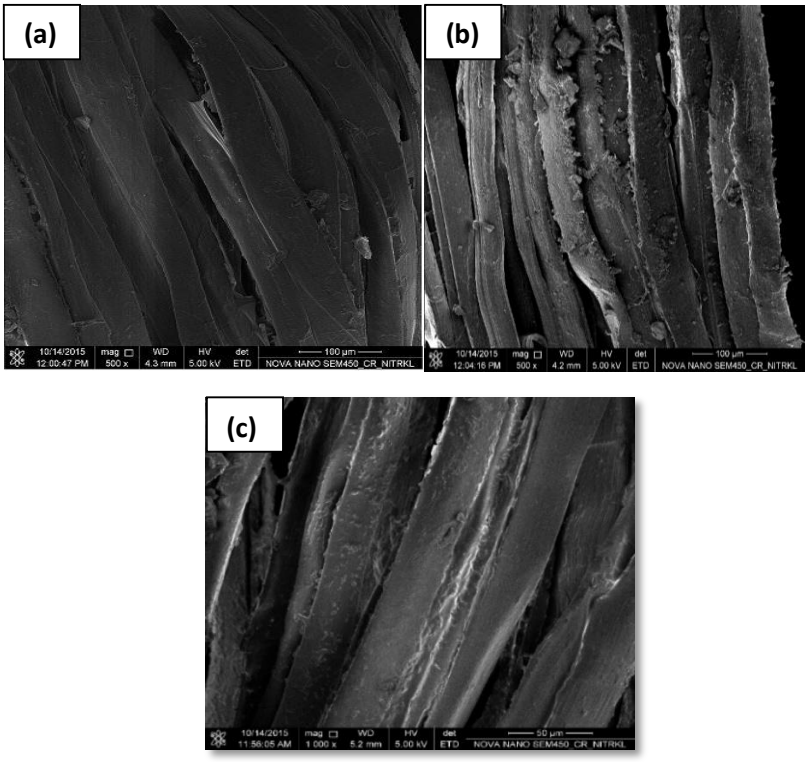


Figure 4.10 (a) Non-coated scaffold (b) Gelatin coated scaffold (c) Chitosan coated

b) Coated scaffold with crosslinkers

The morphology of the scaffolds can clearly be seen in the following images. The surface has a much smoother texture after cross-linking was done. This helps in the attachment of the cells as the cells cannot adhere to rough surfaces. The smooth surface of the scaffold provides a platform for the cell to attach and grow. The images appear to be very similar in nature but on close observation it can be said that the scaffold in which PEG-dimthacrylate has been used (Figure 4.10) as crosslinker is the smoothest. Besides this, when compared with non-coated silk, it can be seen that there is an even coating of biopolymers when coated using crosslinkers. On the other hand, Araldite DY-T crosslinked scaffold was found to be the roughest.

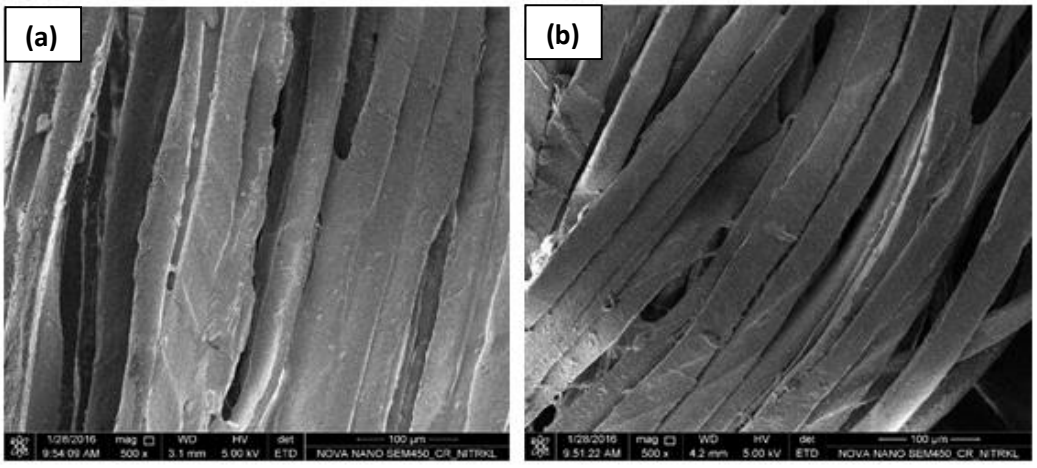


Figure 4.11 (a) PEG-D scaffold (b) Gelatin coated scaffold (c) Chitosan coated

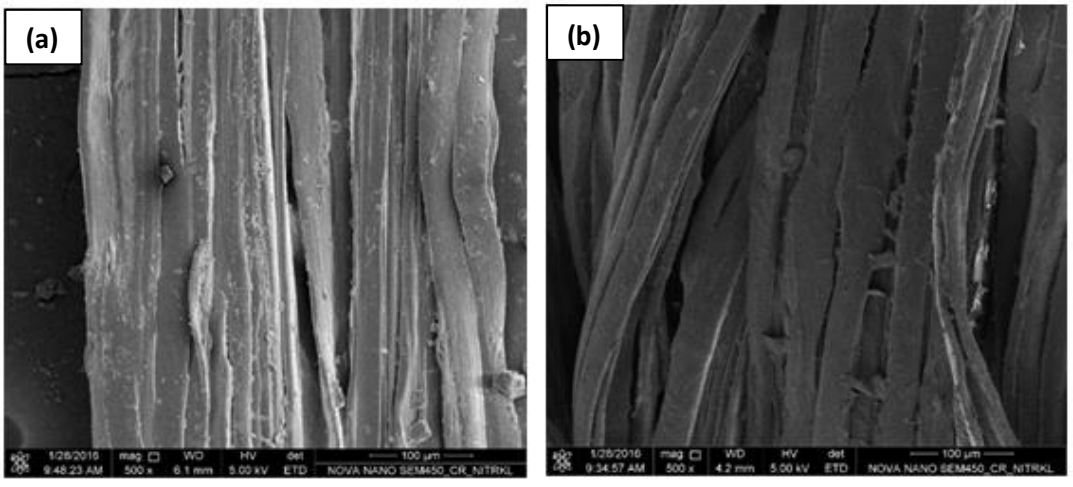


Figure 4.12 Glutaraldehyde cross-linked scaffolds: (a) Gelatin, and (b) Chitosan

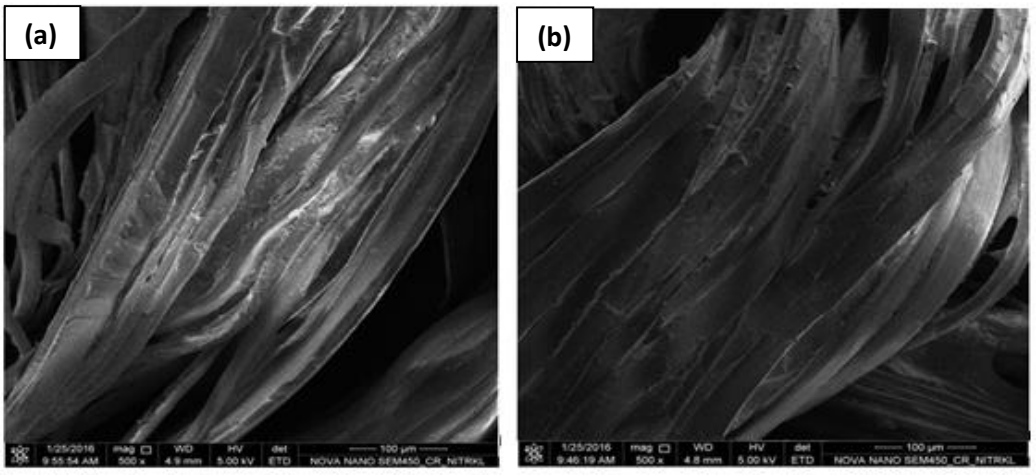


Figure 4.13 Araldite DY-T cross-linked scaffolds: (a) Gelatin and (b) Chitosan

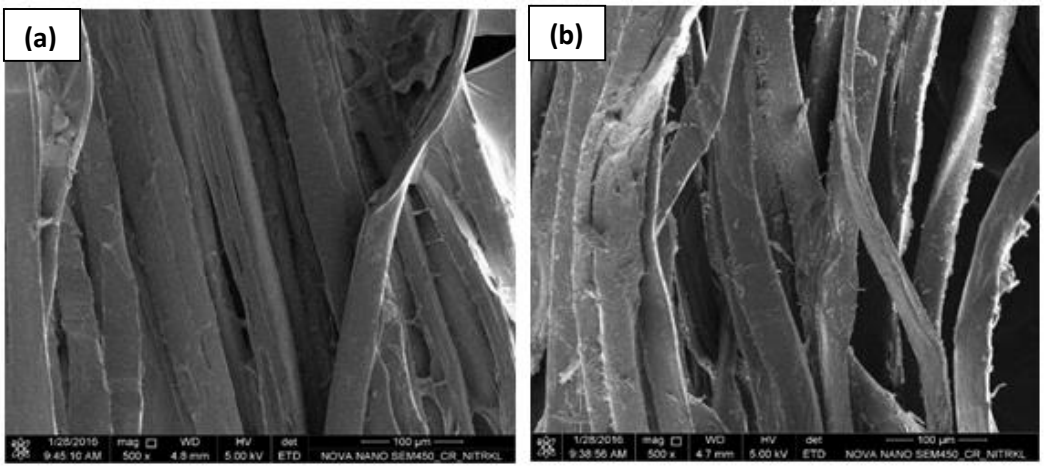


Figure 4.14 Tyrosinase cross-linked scaffolds: (a) Gelatin, and (b) Chitosan

4.2.3 Biodegradation study

The biodegradability of chitosan was found to be the maximum in case of non-crosslinked scaffolds (Figure 4.14) and gelatin was found to be less biodegradable than chitosan. After crosslinking, the rate of biodegradation reduced for all the scaffolds as shown in Figure 4.15. Before crosslinking, chitosan coated scaffold showed biodegradation rate of 25.6% and for gelatin coated scaffolds it was 11.4%. After crosslinking, it reduced to 10.8% for chi-glu ,13% for chi-aral, 21.2% for chi-PEG, 19.7% for chi-tyro, 8.1% for gel-glu, 6.2% for gel-aral, 10.6% for gel-PEG and 9.7% for gel-tyro scaffolds.

A) **Non-crosslinked scaffolds**

	0	3	7	14	21	30
Non-coated scaffold	**0.090**	**0.089**	**0.087**	**0.086**	**0.084**	**0.082**
Chitosan-coated scaffold	**0.090**	**0.088**	**0.082**	**0.077**	**0.071**	**0.068**
Gelatin coated scaffold	**0.090**	**0.089**	**0.085**	**0.083**	**0.081**	**0.079**

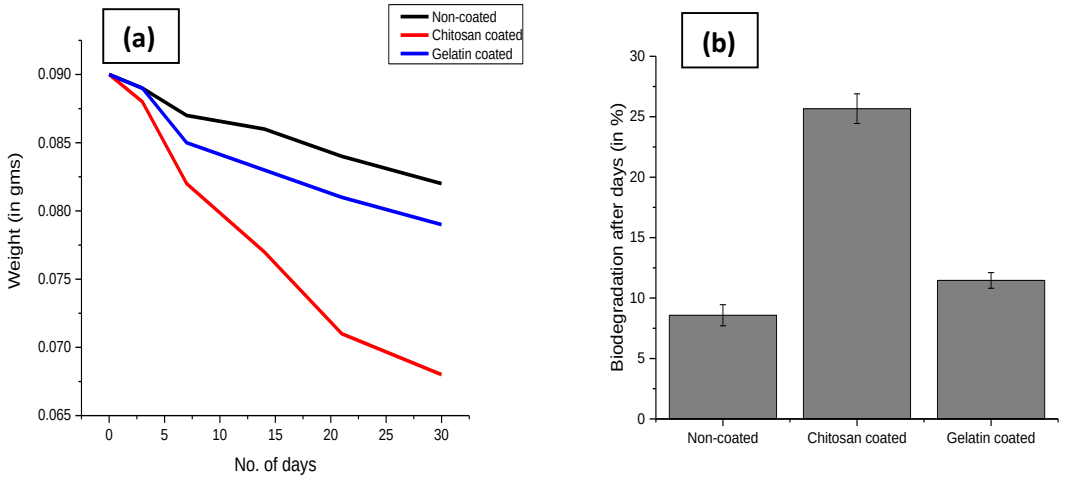


Figure 4.15 a) weight loss (in g) b) weight loss (%)

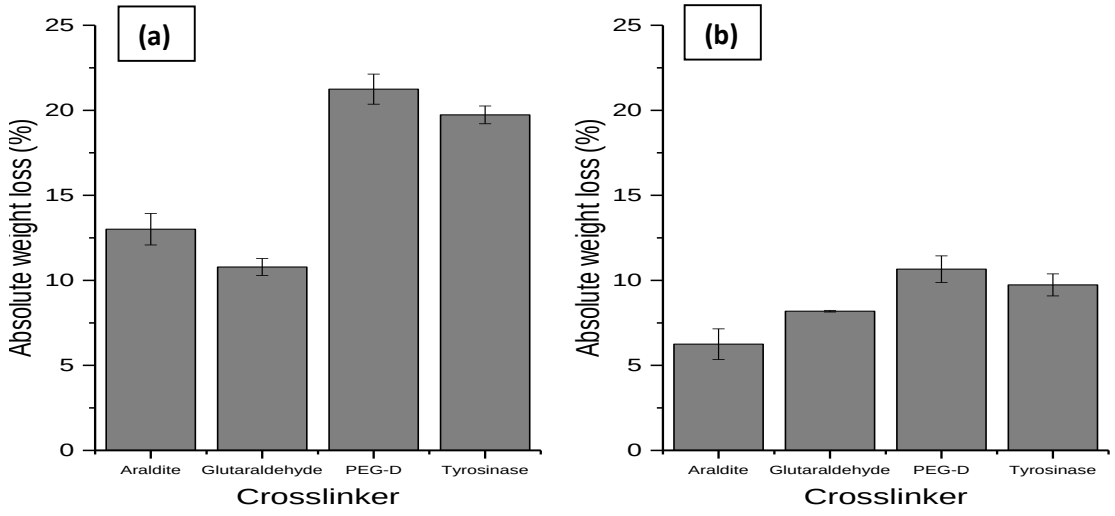


Figure 4.16 Absolute weight loss % after crosslinking a) chitosan b) gelatin

4.2.4 TGA Analysis

From Figure 4.16 a, b and c it can be seen mass loss started at around 100˚C for all the scaffolds. It was observed that even at high temperature of 400˚C no such difference was seen between non-coated and coated scaffolds. This indicated a high thermal stability and the scaffolds did not change their thermal stability even after coating.

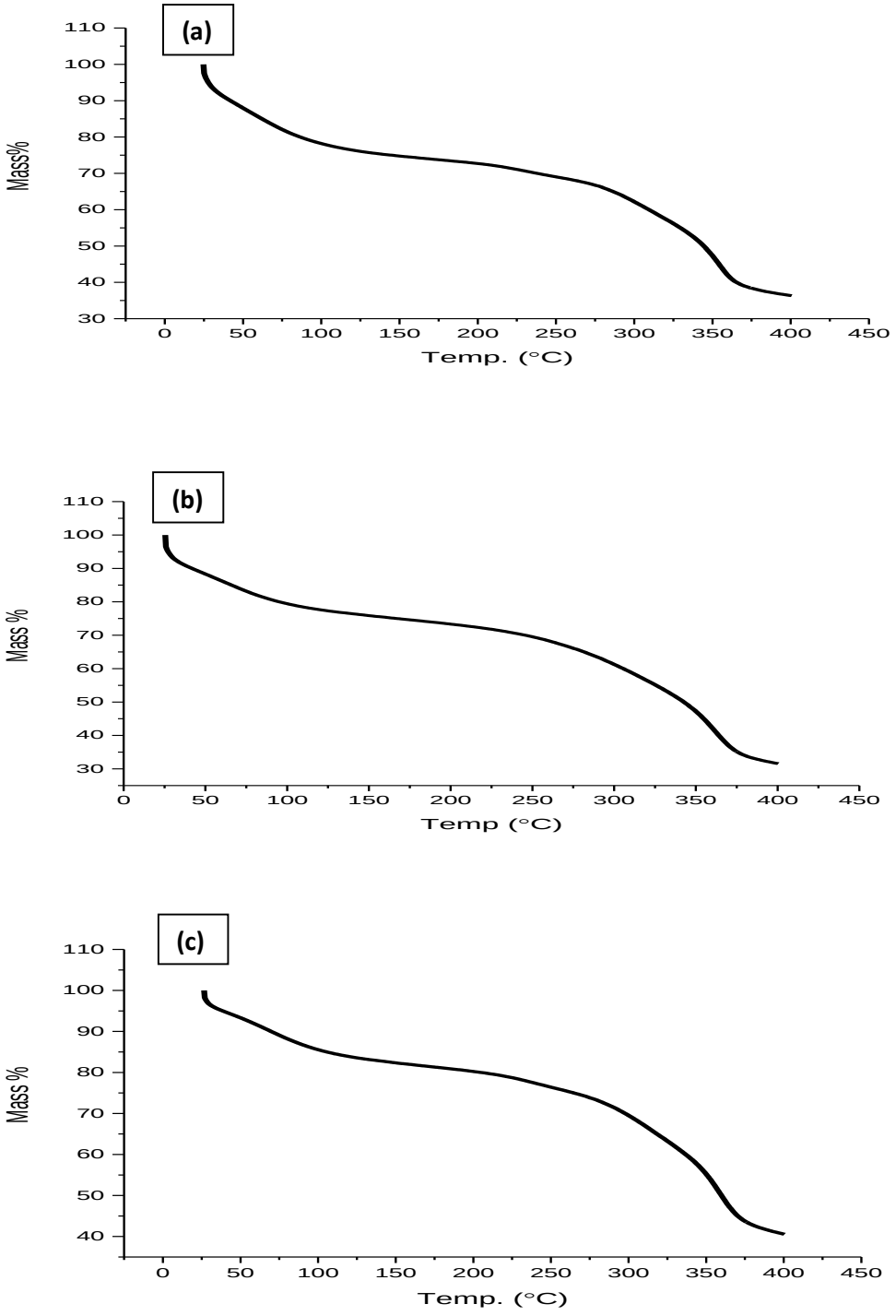


Figure 4.17 Mass loss a) non-coated b) chitosan coated c) gelatin coated

4.2.5 DSC analysis

DSC analysis was done to determine the glass transition temperature of the scaffold. It was observed that neither the melting point nor the glass transition temperature was reached even at high temperature of 400˚C indicating a high thermal stability [Figure 4.17 (a)(b) and(c)]. Besides this, not much difference was observed before and after coating the scaffold. Thus it can be assumed that coating did not change the thermal properties of the scaffold.

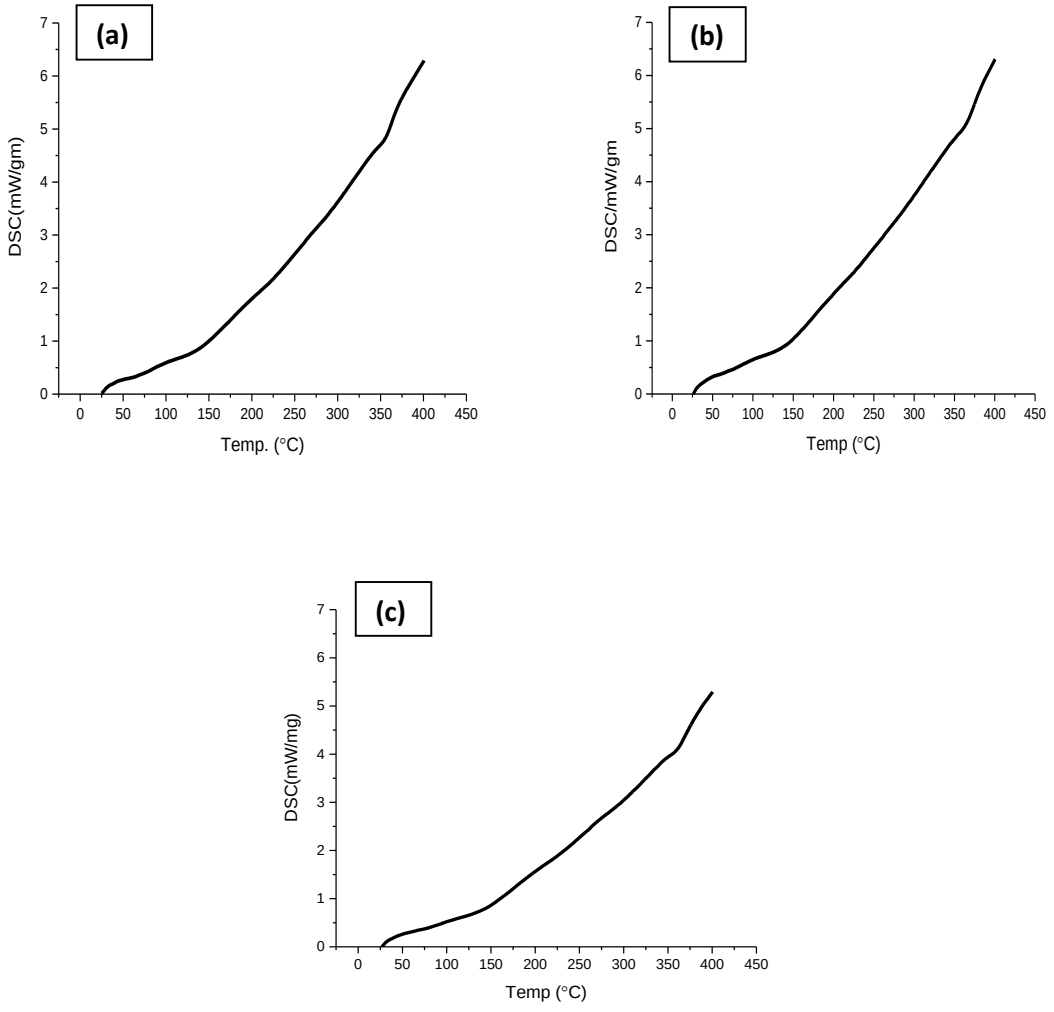
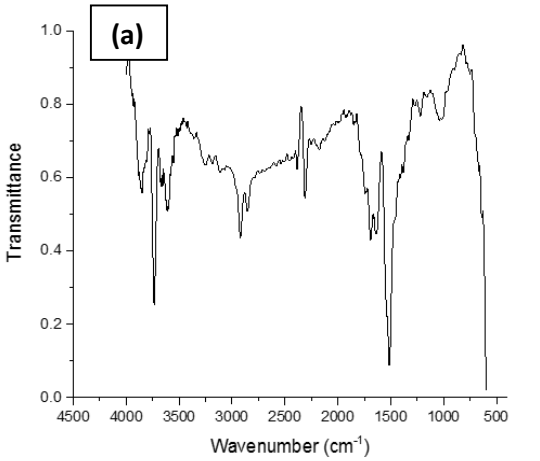


Figure 4.18 DSC analysis a) non-coated b) chitosan coated c) gelatin coated

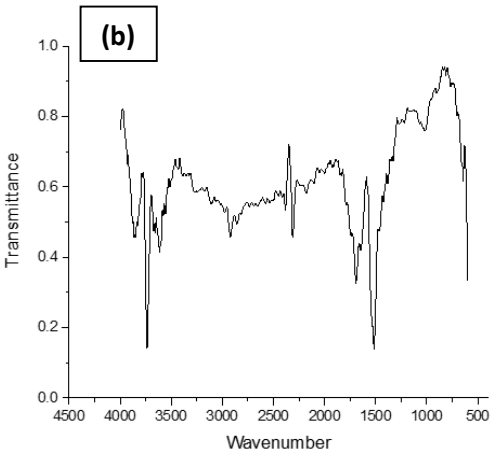
4.2.6 FTIR analysis

In all the figures below, a common peak can be observed at a wavenumber close to 1510 cm^{-1}. This peak represents the presence of amide 2 present in the silk. Scaffolds coated with chitosan and gelatin tend to have a peak of N-H bending vibration at wavenumber close to

1650 cm^{-1}. The sudden change in the peak at around 1075 cm^{-1} denotes the presence of ethoxy group in araldite DY-T. Two immediate peaks at around 2730 and 2850 cm^{-1} represent the aldehydic group of glutaraldehyde. Peaks around 1700 cm^{-1} and 2919 cm-1 represent functional groups for PEG-D and tyrosinase.

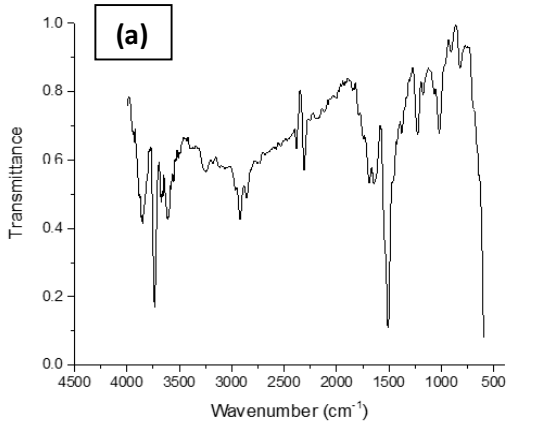


Wavenumber (cm^{-1})	Functional group	Compound
1515	Amide II	Silk
1650	N-H bending vibration	Chitosan
2734 & 2852	Aldehyde	Glutaraldehyde

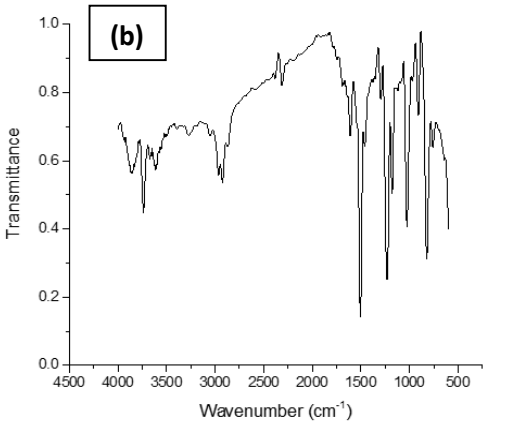


Wavenumber (cm^{-1})	Functional group	Compound
1517	Amide II	Silk
3530	Amide A	Gelatin
2719 & 2868	Aldehyde	glutaraldehyde

Figure 4.19 Glutaraldehyde crosslinked scaffold a) chitosan coated b) gelatin coated

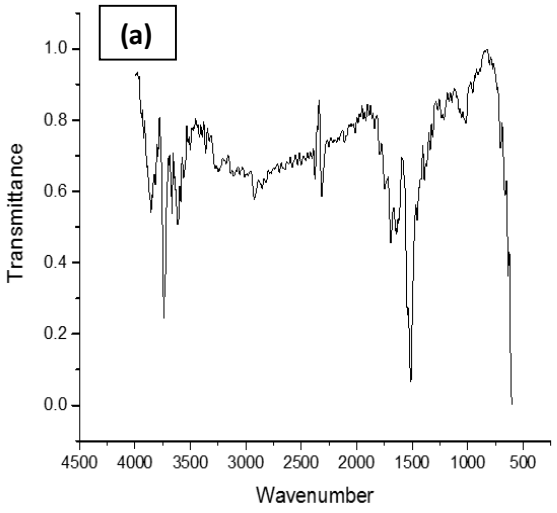


Wavenumber (cm^{-1})	Functional group	Compound
1510	Amide II	Silk
1658	N-H bending vibration	Chitosan
1700 1238 & 1038	C=O C-O	PEG-D

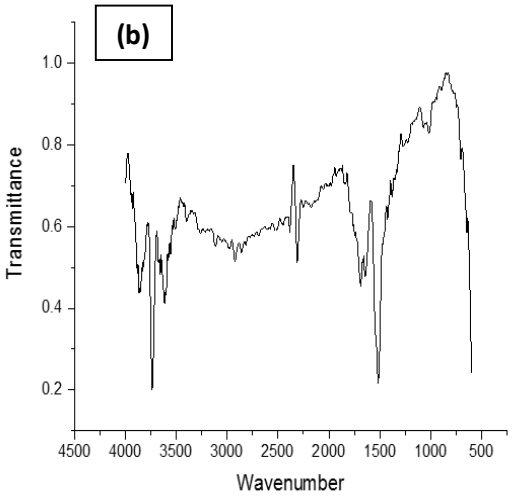


Wavenumber (cm^{-1})	Functional group	Compound
1503	Amide II	Silk
3595	Amide A	Gelatin
1700 1238 & 1038	C=O C-O	PEG-D

Figure 4.20 PEG-dimethacrylate crosslinked scaffold a) chitosan coated b) gelatin coated

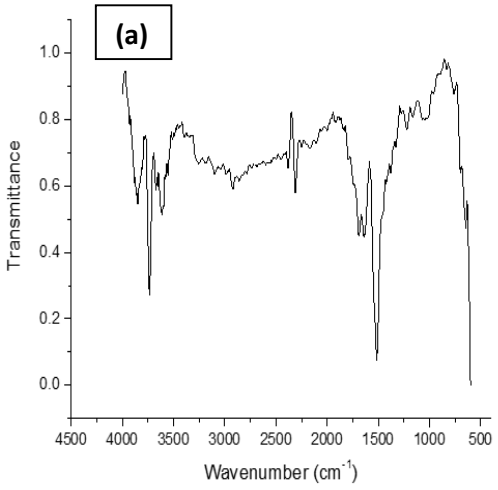


Wavenumber (cm^{-1})	Functional group	Compound
1510	Amide II	Silk
1655	N-H bending vibration	Chitosan
1077	Ethoxy	Araldite DY-T

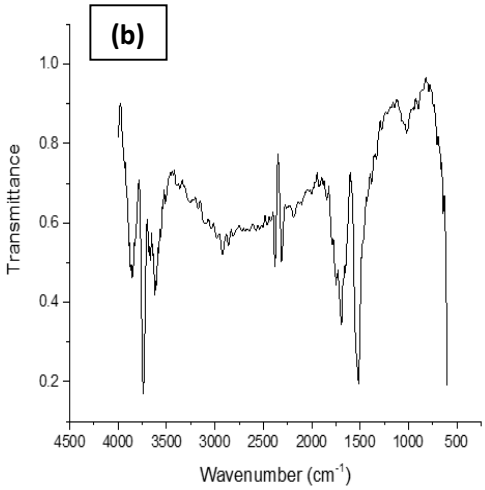


Wavenumber (cm^{-1})	Functional group	Compound
1521	Amide II	Silk
3495	Amide A	Gelatin
1071	Ethoxy	Araldite DY-T

Figure 4.21 Araldite-DY-T crosslinked scaffold a) chitosan coated b) gelatin coated



Wavenumber (cm^{-1})	Functional group	Compound
1516	Amide II	Silk
1655	N-H bending vibration	Chitosan
2919	sp2 C-H	Aromatic



Wavenumber (cm^{-1})	Functional group	Compound
1515	Amide II	Silk
3511	Amide A	Gelatin
2923	sp2 C-H	Aromatic

Figure 4.22 Tyrosinase crosslinked scaffold a) chitosan coated b) gelatin coated

4.2.7 Mechanical Testing

A) Chitosan coated scaffold

The young's modulus of chitosan coated silk was found to be 14.38MPa, tensile strength 8.63MPa and maximum load of 140.91N whereas after 30 days the young's modulus reduced to 4.41 MPa, tensile strength 2.01 MPa and maximum load capacity of 27.76N.

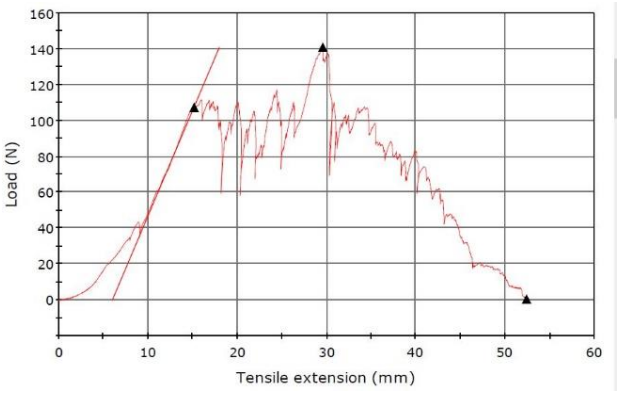


Figure 4.23 Stress-strain curve for chitosan coated scaffold before biodegradation

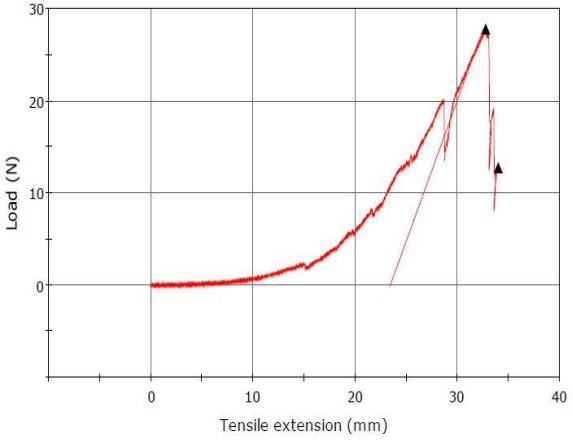


Figure 4.24 Stress-strain curve for chitosan coated scaffold after biodegradation

B) Gelatin coated scaffold

The young's modulus of gelatin coated silk was found to be 12.91 MPa, tensile strength 7.28MPa and maximum load of 74.87 N whereas after 30 days the young's modulus reduced to 9.63 MPa, tensile strength 5.68MPa and maximum load capacity of 25.38N.

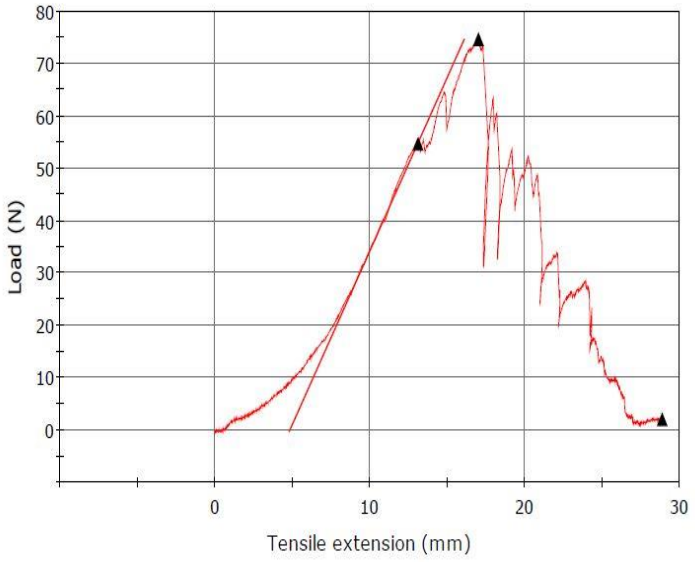


Figure 4.25 Stress-strain curve for gelatin coated scaffold before biodegradation

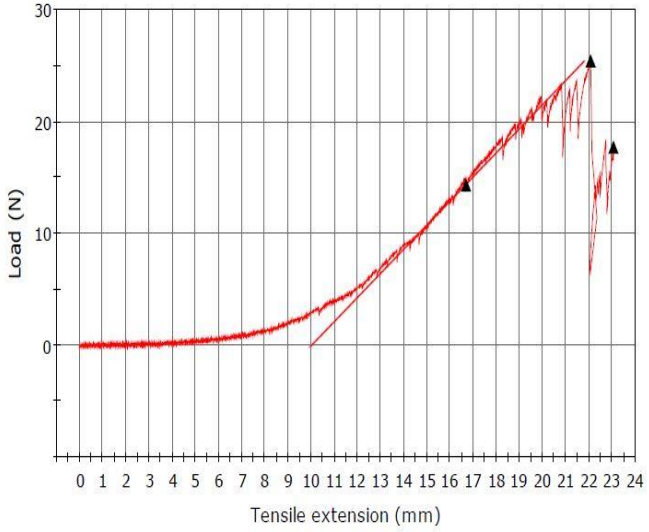


Figure 4.26 Stress-strain curve for gelatin coated scaffold after biodegradation

4.3 Cell viability assay

MTT assay was done for the cells growing on the coated scaffold. The cells were incubated for 3 hours with the dye. The result showed clearly that the scaffold provided a conducive microenvironment for the cells to grow. All the scaffolds favoured the growth of cells and finally it was confirmed by MTT assay that cells can be grown on the fabricated scaffold with no further modifications.

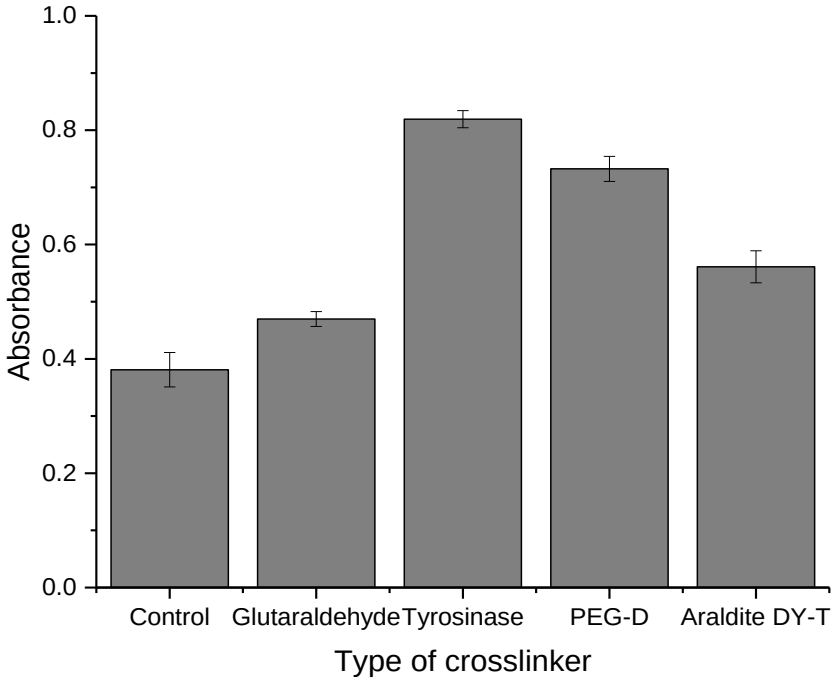


Figure 4.27 MTT assay of gelatin coated scaffold with different crosslinkers

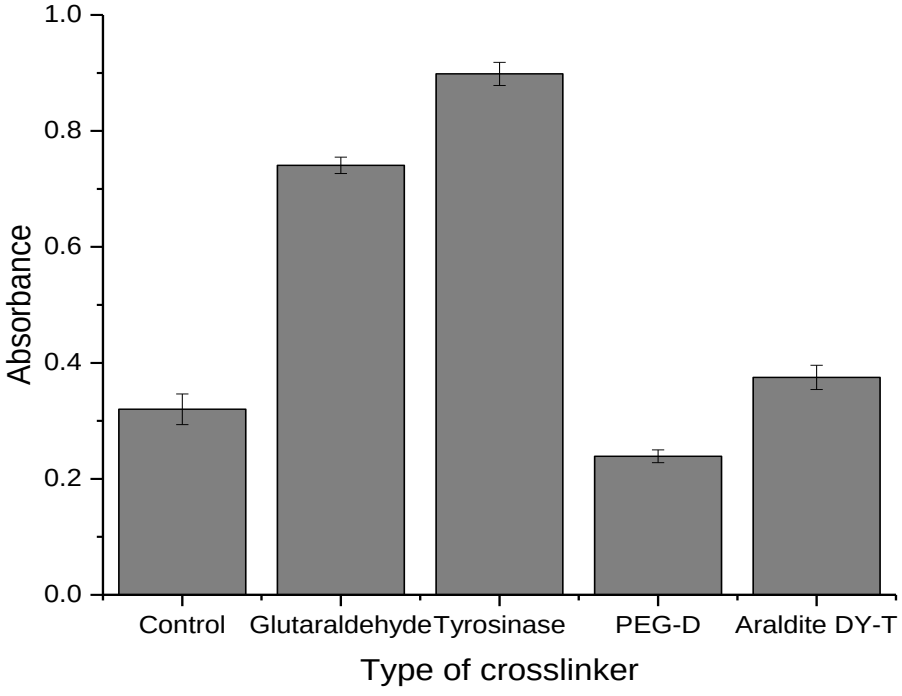


Figure 4.28 MTT assay of chitosan coated scaffold with different crosslinkers

It is well-known that more the absorbance more the number of viable cells. From the above result, it can infer that all the scaffolds support the growth of cells. For gelatin coated scaffold, PEG-dimethacrylate was found to have the maximum absorbance and glutaraldehyde had the least absorbance whereas, for chitosan coated scaffolds, tyrosinase showed the maximum and PEG-dimethacrylate showed the least absorbance.

Chapter 5

5. Summary and Conclusion

5.1 Summary

- The coating efficiency of the biopolymer on to the scaffold increased after cross-linking. For gelatin coated scaffolds, PEG -dimethacrylate crosslinked scaffolds were found to have the maximum efficiency (from 9.1% to 22.9%), whereas in case of chitosan coated scaffolds, Araldite DY-T (from 4.5% to 19.5%) showed maximum efficiency
- Except tyrosinase, all other crosslinkers reduced water absorption percentage.
- The surface smoothness of the crosslinked scaffolds was much more than the non-crosslinked scaffolds as observed from FE-SEM analysis.
- The biodegradation rate of the scaffolds reduced after cross-linking. Before crosslinking, chitosan coated scaffold showed biodegradation rate of 25.6% and for gelatin coated scaffolds it was 11.4%. After crosslinking, it reduced to 10.8% for chi-glu ,13% for chi-aral, 21.2% for chi-PEG, 19.7% for chi-tyro, 8.1% for gel-glu, 6.2% for gel-aral, 10.6% for gel-PEG and 9.7% for gel-tyro scaffolds.
- DSC and TGA analysis showed that not much difference was found in the scaffolds before and after cross-linking. The scaffolds were thermally stable even at higher temperatures.
- FTIR analysis confirmed the presence of functional groups corresponding to the biopolymers and the crosslinkers in the respective scaffolds even after washing of the scaffolds.
- The stiffness of chitosan coated scaffold and gelatin coated scaffold reduced by 69% and 25% respectively over a period of 30 days (Chitosan from 14.38MPa to 4.41MPa and Gelatin from 12.91MPa to 9.63MPa)
- MTT assay showed that the scaffolds provide a conducive microenvironment for the cells to grow. For gelatin coated scaffold, PEG-dimethacrylate was found to have the maximum absorbance and glutaraldehyde had the least absorbance whereas, for chitosan coated scaffolds, tyrosinase showed the maximum and PEG-dimethacrylate showed the least absorbance at 590nm.

5.2 Conclusion

Ligament injury is very hard to recover on its own due to very low vascularity. Tissue engineering is a great tool for the regeneration of the ligament. The major drawback in tissue engineering is the failure of the ligament to integrate with the bone resulting in improper development which may finally end up in slipping of the ligament. The main reason for this is underdevelopment of the enthesis region. The current study concentrated on the fabrication of scaffold using knitted silk as a backbone coated with chitosan or gelatin. To enhance the coating efficiency, various types of crosslinkers were used which successfully enhanced the coating efficiency. This was followed by various characterization methods like swelling studies, biodegradation studies, DSC & TGA analysis, FTIR analysis and mechanical studies which determined that these scaffolds can be used for tissue engineering applications. Finally, the MTT assay was done which also showed that these scaffolds are capable of allowing the cells to grow on them.

5.3 Future Work

The native ligament present inside human body lacks vascularity. So, the major target would be to inhibit angiogenesis. To inhibit angiogenesis, different kinds of agents are available. Besides this, the drug release testing will also play a major role regarding the future studies related to this project. Finally, in-vivo testing will be of prime importance as the scaffold will be implanted inside the body.

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